COMPOUNDS AND METHODS FOR PHARMICO-GENE THERAPY OF EPITHELIAL SODIUM CHANNEL ASSOCIATED DISORDERS

Cross-Reference to Related Applications

The present application claims the benefit of the filing date of U.S. application Serial No. 60/459,323, filed March 31, 2003, and U.S. application Serial No. 60/512,347, filed October 16, 2003, the disclosures of which are incorporated by reference herein.

Statement of Government Rights

This invention was made at least in part with a grant from the Government of the United States of America (HL58340) from the National Institutes of Health). The Government may have certain rights in the invention.

Background of the Invention

Cystic fibrosis (CF) is caused by a genetic mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), and is the most common genetic disorder in the Caucasian population. CFTR is a chloride channel that localizes to the apical membrane of epithelial cells in many organs such as the lung. The channel is activated by cyclic AMP (cAMP) and regulated by PKA- and PKC-dependent phosphorylation. In addition to functioning as a chloride channel, CFTR has also been shown to regulate several other ion channels at the cell surface (Jiang et al., 1998), including the epithelial amiloride-sensitive sodium channel (ENaC) (Stults et al., 1997; Donaldson et al., 2002), outward rectifying chloride channel (ORCC) (Gabriel et al., 1993; Schwiebert et al., 1998), renal potassium channel (ROMK2) (Cahill et al., 2000), and the calcium activated chloride channel (Kunzelmann et al., 1997).

Several mechanisms have been proposed to account for the increased bacterial colonization seen the CF lung (Jiang et al., 1998). These include the loss of CFTR function leading to dysregulated enhancement of ENaC sodium currents, which is proposed as a predominant mechanism for airway dehydration and poor mucociliary clearance the CF lung (Knowles et al., 2002; Guggino et al., 1999). Alternative mechanisms of pathogenesis also include altered ionic composition of

the surface airway fluid which reduces the activity of innate immune defenses in the CF airway as a result of CFTR dyfunction (Jiang et al., 1998; Guggino et al., 1999; Smith et al., 1996).

Traditional treatments for CF include chest physiotherapy (e.g., percussion and postural drainage), various broncodilators, nutritional supplements (e.g., pancreatic enzymes and vitamins), exercise and rehabilitation, and long-term oxygen therapy for chronic hypoxemia. However, numerous clinical trails for cystic fibrosis have evaluated pharmacologic approaches to correct primary defects associated with CFTR dysfunction. These have included approaches to enhance endogenous mutant CFTR function at the apical membrane (Ahrens et al., 2002; Rubenstein et al., 1998) and normalization of ENaC function or airflow using aerosolized amiloride (Knowles et al., 1990; Graham et al., 1993; Pons et al., 2000; U.S. Patent Nos. 4,501,729 and 4,866,072). More recently, recombinant adeno-associated virus (rAAV) has been used to delivery a functional CFTR cDNA to the maxillary sinus and/or lungs of CF patients (Aitken et al., 2001; Flotte et al., 2003; Wagner et al., 2002; Wagner et al., 1999). Although recent trials with rAAV-2 have demonstrated an impressive safety profile and long-term persistence of vector genomes in the airway epithelia of the lung, successful transduction of airway cells as measured by vector derived CFTR mRNA have not been optimal possibly due to the relatively poor binding to the surface of polarized human airway cells (Zabner et al., 2000), and the fact that known rAAV-2 receptors do not reside on the apical surface of the human airway (Duan et al., 1998). Such findings have led to the development and application of alternative serotypes such as rAAV-5 that bind to alternative receptors at the airway surface (Walters et al., 2001) and have enhanced transduction efficiencies (Zabner et al., 2000).

Alternative developments evaluating intracellular barriers to rAAV transduction in the human airway have suggested that both rAAV-2 and rAAV-5 are susceptible to ubiquitin/proteasome interactions which modulate the ability of virions to complete their life cycle and efficiently traffic to the nucleus (Duan et al., 2000). These studies have led to the application of proteasome inhibitors/modulating agents capable of significantly enhancing transduction of both serotypes from the apical membrane (Duan et al., 2000; Ding et al., 2003).

Interestingly, studies directly comparing rAAV-2 to rAAV-2/5 vectors have suggested that the maximal potential of rAAV-2 to transduce airway epithelial from the basolateral membrane is an order of magnitude higher than rAAV-2/5 infection from the apical or basolateral membrane (Ding et al., 2003). These findings suggest the possibility that if apical barriers to infection could be fully circumvented, rAAV-2 may emerge as a preferential vector for gene therapy of the CF lung.

Additional limitations to rAAV-mediated gene delivery of CFTR include the limited packaging capacity of this vector (about 5 kb) and the relatively large size of the CFTR cDNA (4.5 kb). Several strategies have been used to fit the CFTR cDNA into rAAV vectors including the use of the ITR as a promoter (Flotte et al., 1993) and the deletion of regions of CFTR thought not to be necessary for *in vivo* function (Zhang et al., 1998; Ostedgaard et al., 2002). The first strategy is currently used in clinical trails with rAAV-2 based vectors. Although *in vitro* studies have clearly demonstrated the ability of the ITR to function as a promoter (Flotte et al., 1993), expression is much lower than that from heterologous promoters (Duan et al., 2000b). Hence, the low activity of the ITR as a promoter is currently thought to be one of the major reasons for lack of detectable vector derived CFTR mRNA expression in clinical trails (Flotte et al., 2003).

Thus, there exists a need to identify agents useful to inhibit or treat conditions or disorders in the airway which are associated with one or more channel proteins.

Summary of the Invention

The invention provides a method to identify an agent, or a combination of agents, that alters ENaC activity in a eukaryotic cell, e.g., a mammalian cell such as a mammalian lung, kidney or colon cell, or a population of eukaryotic cells, e.g., in tissues or organs. The method comprises contacting the cell or population of cells with the one or more agents and determining whether the level or amount of ENaC is altered. In one embodiment, the cell or population of cells are epithelial cells such as airway epithelial cells. In another embodiment, the cell or cells are kidney tubule, e.g., distal nephron including distal convoluted tubule, connecting tubule, and cortical and medulary collecting duct, skin, liver, bladder, colon, sweat gland,

mammary gland, salivary gland, placenta or uroepithelium cells. Preferred cells include those of mammals, birds, fish, and reptiles, especially domesticated mammals and birds such as humans, non-human primates, cattle, sheep, pigs, horses, dogs, cats, mice, rats, rabbits, chickens, and turkeys. For example, polarized human airway epithelial cells grown at an air-liquid interface or human bronchial xenografts are useful to identify agents which inhibit or decrease the level or amount of ENaC. The agents of the inventions may be contacted with any cell comprising native or recombinant ENaC, e.g., cell membrane bound ENaC. It is envisioned that agents identified as inhibiting the level or amount of ENaC may have variations in the degree of inhibition, time course and/or duration of inhibition, cell or tissue type specificities and/or the concentration employed for inhibition.

For example, the invention provides a method to identify one or more agents that inhibit or decrease the level or amount of ENaC, e.g., agents including but not limited to those that inhibit transcription of one or more ENaC subunit genes, alter the level, amount or activity of a molecule that alters ENaC transcription, alter ENaC RNA stability, and/or alter the trafficking and processing of molecules, for instance, molecules of non-viral origin through intracellular compartments, including without limitation proteasomes, endosomes, and trans-golgi, and/or through the cytosol, e.g., via cytoskeletal components such as microtubules or microfilaments. In one embodiment, the agent is not an antagonist of ENaC. In another embodiment, the agent is not an agent that binds a cell membrane bound protein, e.g. ENaC or the receptor for hepatocyte growth factor. In yet another embodiment, the agent is not an agent that alters post-translational processing of ENaC. In another embodiment, the agent is not a gene of, or a gene product encoded by, a mammalian genome, e.g., a protein encoded by a mammalian cell, the complement of the gene, or a portion of the gene or its complement, e.g., an antisense oligonucleotide.

An agent or library of agents, e.g., chemical libraries including peptide libraries, may be randomly screened in the methods of the invention. Agents to be tested may be selected from agents having desirable properties for a particular cell type, tissue type or disease type to be treated. Alternatively, agents to be tested are

selected from agents including those having desirable properties, e.g., therapeutic properties, for instance, agents in clinical trials or having FDA approval or functional and/or structural properties of agents identified as inhibiting or decreasing the level or amount of ENaC. In one embodiment, agents may be selected from agents that modulate the proteasome, e.g., agents including but not limited to those that bind to a proteasome, alter one or more activities of a proteasome, e.g., inhibit the proteolytic activity of the proteasome, alter subcellular positioning or trafficking of the proteasome, alter the interaction of one or more molecules with a proteasome, or stabilize the proteasome. Proteasomes are the main proteolytic complex in the cytosol and nucleus, and can be transported between the cytoplasm and nucleus. For instance, the 26S proteasome complex comprises a 19S regulatory unit and a 20S catalytic core which has chymotrypsin-like activity, i.e., cleavage after large hydrophobic residues, trypsin-like activity, i.e., cleavage after basic residues, post-glutamyl hydrolase activity, i.e., cleavage after acidic residues, branched amino acid cleavage activity and small neutral amino acid cleavage activity.

Thus, in one embodiment, chemical libraries are selected based on chemical structures known to interact with the proteasome, or other intracellular processing pathways, e.g., endosomal compartments. In another embodiment, agents are selected from chemotherapeutics, antibiotics, lipid lowering agents or food additives. Antibiotics include but are not limited to macrolides, penicillins, quinolones, sulfonamides and tetracyclines, e.g., cephalosporins, bacitracin, vancomycin, ristocetin, erythromycin, oleandomycin, carbomycin, spiramycin, lincomycin, clindamycin, chlortetracycline, minocycline, oxytetracycline, streptomycin, amikacin, gentamycin, kanamycin, neomycin, tobramycin, polymyxins, nystatin, amphotericin B, mitomycin, actinomycin, nalidixic acid, novobiocin, griseofulvin, rifampicins, and trimethoprim. Chemotherapeutics include but are not limited to anti-fungal agents, anti-bacterial agents, antiviral agents, e.g., nucleoside analogs, phosphonoacetate, phosphonoformate, amantadine, rimantadine, enviroxime, 4',6-dichloroflavan, chalcone Ro 09-0410, arildone, disoxaril, 3'-azidothymidine, suramin and HPA 23, and anticancer agents, e.g.,

alkylating agents, antimetabolites, plant alkaloids, antitumor antibiotics and steroid hormones such as cyclophosphamide, nitrosoureas, carmustine (BCNU), lomustine (CCNU), 6-mercaptopurine, 5-fluoroouracil (5FU), doxorubicin (adriamycin), mitomycin-C, bleomycin, vincristine, vinblastine, and tamoxifen.

As described herein, to directly compare rAAV-2 or rAAV-2/5 vectors for their ability to correct the CFTR detect in polarized CF airway epithelia, several full-length CFTR cDNA rAAV vectors were prepared. The vectors were packaged in either type 2 and 5 capsids, and following apical infection of polarized CF airway epithelia, analyzed for their ability to correct both Na hyperabsorption and Cl transport defects which accompany the CF phenotype, and for their efficiency of transduction in the presence or absence of proteasome modulating agents (LLnL/Doxorubicin). In particular, the identical ITR-CFTR vector used in clinical trials for CF (AVtgCF) was compared to that of a vector harboring a minimal 83 bp promoter directing expression of the full-length CFTR cDNA (AVCF83). The comparison included measurements of short circuit current, quantitative RS-PCR, and TaqMan DNA PCR, so as to quantify functional correction of CFTR chloride currents, vector-derived mRNA, and vector DNA, respectively. The data demonstrated that rAAV-2 based vectors are more efficacious than rAAV-2/5 at expressing CFTR-derived mRNA and correcting CFTR chloride transport abnormalities in the presence of applied proteasome modulating agents.

Interestingly, the application of proteasome modulating agents at the time of infection not only improved the functional conversion of rAAV genomes to expressible forms but also reduced the ENaC hyperabsorption CF phenotype in a manner independent of CFTR gene expression. Quantitative RT-PCR demonstrated that the addition of proteasome modulating agents reduced γ-ENaC subunit mRNA levels in polarized CF airway epithelia by 15-fold. The long-term (15 day) persistence of this effect on ENaC activity correlated with doxorubicin-dependent CpG methylation of the γ-ENaC promoter. These unexpected findings demonstrate for the first time the identification of a new class of dual therapeutic agents capable of both treating primary defects of a disease while enhancing gene therapy of the disorder. In particular, these findings suggest that in addition to improving rAAV

transduction, modulation of the proteasome also significantly attenuates ENaC sodium hyperabsorption defects in CF airway epithelia. These studies, which provide the first demonstration of rAAV-mediated CFTR functional correction in CF polarized airway epithelial, suggest that proteasome modulating agents may have dual therapeutic potential for both enhancing rAAV transduction and ameliorating fluid transport defects in CF caused by dysregulated ENaC. For instance, agents which alter ENaC activity may be screened for their ability to alter fluid transport or absorption in polarized airway epithelial cells. In one embodiment, one or more agents and, optionally a dye, such as a fluorescent dye, in a small volume of liquid, are contacted with polarized airway epithelial cells, and the presence or amount of the dye and/or the amount (depth) of extracellular liquid in the treated cells is detected or determined, e.g., using a confocal microscope, and compared to untreated cells. In addition, agents which alter ENaC activity may be screened for their association with the methylation of other promoters, which may result in the identification of agents that are associated with the methylation of more than one promoter as well as agents that are associated with the methylation of only one promoter.

The identification of agents with dual therapeutic action may be extremely useful in clinical trials for CF lung disease as well as other diseases. Thus, one or more agents identified by the methods may provide clinically useful strategies for *in vivo* therapy, e.g., gene therapy of respiratory disorders such as cystic fibrosis and others associated with inflammation, e.g., due to infection with a pathogen, for instance, bacterial infection, and including conditions associated with aberrant, e.g., increased, ENaC activity. For instance, the amount of fluid absorption or transport in the lung may be linked to bacterial clearance, and aerosolized delivery of one or more agents of the invention, which alter ENaC activity to a mammal, may alter (e.g., modulate) the inflammatory response, resulting in enhanced clearance or decreased inflammation.

The data also showed that vectors harboring a short 83 bp minimal promoter improved functional correction and the transcriptional activity of vector genomes by 30% as compared to ITR promoter driven vectors.

The invention provides a method to identify one or more agents that decrease the level or amount of transcription of one or more subunits of ENaC in mammalian cells. The method includes contacting mammalian cells which express ENaC with at least one agent that is a proteasome modulating agent, wherein the agent is not a gene or gene product encoded by the genome of the cells, the complement of the gene, or a portion of the gene or its complement, and identifying whether an agent decreases the level or amount of transcription from one or more subunits of ENaC in the mammalian cells.

Also provided is a method to identify one or more agents that decrease the level or amount of transcription from the α, β, and γ subunits of ENaC in mammalian cells. The method includes contacting mammalian cells which express ENaC with the one or more agents and identifying one or more agents that decrease the level or amount of transcription from the α, β, and γ subunits of ENaC in the mammalian cells. In one embodiment, the agent is not a gene or a gene product encoded by the genome of the cells, the complement thereof, or a portion thereof.

Further provided is a method to identify one or more agents that decrease the level or amount of transcription of one or more subunits of ENaC in mammalian cells. The method includes contacting mammalian cells which express ENaC with at least one agent that enhances viral transduction and identifying one or more agents that decrease the level or amount of transcription from one or more subunits of ENaC in the mammalian cells. In one embodiment, the agent is not a gene or gene product encoded by the genome of the cells, the complement thereof, or a portion thereof.

A method to identify one or more agents with dual therapeutic activity is also provided. In one embodiment, the method includes selecting one or more agents which inhibit or treat one or more symptoms of a disease which is associated with aberrant expression or activity of ENaC, contacting mammalian cells with the one or more agents and a gene therapy vector, and identifying an agent that enhances the efficacy of the gene therapy vector relative to mammalian cells contacted with the gene therapy vector but not contacted with the one or more agents. In another embodiment, the method includes selecting one or more agents that enhance the efficacy of a gene therapy vector in mammalian cells, contacting

mammalian cells having aberrant expression or activity of ENaC with the one or more agents, and identifying an agent that alters ENaC expression or activity.

Agents of the invention may be used alone or in combination to produce additive or synergistic effects, e.g., to alter the level or amount of ENaC, to inhibit reabsorption of salts and water from mucous secretions in a tissue or organ, e.g., in the lung, to hydrate mucous secretions in a tissue or organ, to increase airway surface liquid volume, e.g., in the lung, to facilitate mucous clearance in a tissue or organ, to inhibit or treat conditions associated with aberrant ENaC activity, for instance, cystic fibrosis, Liddle's syndrome, hypertension, pain, and pulmonary edema, as well as chronic bronchitis, asthma, and acute lung injury. For CF, the agents of the invention may be employed with mineral corticoid receptor antagonists, glucocorticoid receptor antagonists, pyrazine diuretics, pyrazinoyl guanidine sodium channel blockers, amiloride, benzamil, phenamil, lanthione antibiotics, nucleotides or dinucleotides, as well as nucleic acids or oligonucleotides; viral gene transfer vectors (including adenovirus, adeno-associated virus, and retrovirus gene transfer vectors); enzymes; and hormone drugs or physiologically active proteins or peptides such as insulin, somatostatin, oxytocin, desmopressin, leutinizing hormone releasing hormone, nafarelin, leuprolide, adrenocorticotrophic hormone, secretin, glucagon, calcitonin, growth hormone releasing hormone, growth hormone, and the like. Enzyme drugs that may be used to carry out the present invention, include but are not limited to DNAse (for the treatment of, e.g., cystic fibrosis), α_1-antitrypsin (e.g., to inhibit elastase in the treatment of emphysema), etc. Suitable anti-inflammatory agents, including steroids, for use in the methods of the present invention include, but are not limited to, beclomethasone dipropionate, prednisone, flunisolone, dexamethasone, prednisolone, cortisone, theophylline, albuterol, cromolyn sodium, epinephrine, flunisolide, terbutaline sulfate, alpha-tocopherol (Vitamin E), dipalmitoylphosphatidylcholine, salmeterol and fluticasone dipropionate. Examples of antibiotics that may be employed include, but are not limited to tetracycline, choramphenicol, aminoglycosides, for example, tobramycin, beta-lactams, for example ampicillin, cephalosporins, erythromycin and derivatives thereof, clindamycin, and the like. Suitable anti-viral agents include acyclovir, ribavirin,

ganciclovir and foscarnet. Suitable anti-neoplastic agents include, but are not limited to, etoposid, taxol, and cisplatin. Antihistamines include, but are not limited to, diphenhydramine and ranitadine. Anti-*Pneumocystis carinii* pneumonia drugs such as pentamidine and analogs thereof may also be used. Anti-tuberculosis drugs such as rifampin, erythromycin, chlorerythromycin, etc. Chelators of divalent cations (e.g., EGTA, EDTA), expectorants, and other agents useful in the loosening of mucous secretions (e.g., n-acetyl-L-cysteine) may also be administered as desired in the practice of the present invention.

Cells, tissues, organs or organisms may be contacted with one or more agents of the invention simultaneously or sequentially, at a single time point or at multiple time points. One of ordinary skill in the art will appreciate that the manner and timing of agent administration will be influenced by the duration and degree of inhibition of the agent, pharmaceutical properties of the agent, and underlying disease condition of the affected tissue, organ or organism.

Agents identified by the method of the invention may be also particularly useful in conjunction with or to potentiate gene therapy that employs nucleic acid-based vectors, e.g., viral vectors, to introduce and/or express a therapeutic peptide or polypeptide in cells of an animal, e.g., a mammal. The agents are also useful in conjunction with nucleic acid-based vaccine vectors to introduce and/or express an immunogenic prophylactic polypeptide or peptide, such as one from a virus, fungus, bacterium, yeast or cancer cell, so as to induce an immune response to that polypeptide or peptide in an animal administered the nucleic acid-based vector. Further, cells may be contacted with one or more agents prior to nucleic acid-based therapy, concurrently with nucleic acid-based therapy, subsequent to nucleic acid-based therapy, or any combination thereof. For instance, agents of the invention may be employed with gene therapy vectors, e.g., viral vectors such as adenovirus vectors, herpes virus vectors, lentivirus vectors, retroviral vectors and/or rAAV vectors. For example, the dual activities of certain agents of the invention may potentiate, or be employed in conjunction with, gene therapy vectors to decrease the dose or total number of molecules, e.g., viral particles, employed to achieve an efficacious result, increase the gene transfer, e.g., transduction, frequency, and/or

for viral vectors, broaden the serotype infectivity pattern, and/or alter the *in vivo* microenvironment to allow increased availability of viral binding. The use of agents of the invention to both treat primary pathophysiologic defects of a disease and potentiate gene therapy vectors is also referred to as pharmico-gene therapy. In one embodiment, the vector is not an rAAV vector. The gene being expressed in the vector can be either a DNA segment encoding a polypeptide, with whatever control elements (e.g., promoters, operators) are desired, or a non-coding DNA segment, the transcription of which produces all or part of some RNA-containing molecule (such as a transcription control element, +RNA, or anti-sense molecule). In particular, therapeutic genes useful in such vectors include ones that encode a functional peptide or polypeptide. A "functional" peptide or polypeptide is one which has substantially the same activity as a reference peptide or polypeptide, for example, a wild-type (full-length) peptide or polypeptide. For example, therapeutic genes useful in the vectors of the invention include but are not limited to the β-globin gene, the γ-globin gene, Factor VIII gene, Factor IX gene, the erythropoietin gene, the cystic fibrosis transmembrane conductance regulator gene (CFTR), the dystrophin gene, the Fanconi anemia complementation group, a gene encoding a ribozyme, an antisense gene, a low density lipoprotein (LDL) gene, a tyrosine hydroxylase gene (Parkinson's disease), a glucocerebrosidase gene (Gaucher's disease), an arylsulfatase gene (metachromatic leukodystrophies), as well as genes encoding immunogenic polypeptides or peptide, such as those useful for vaccines, or genes encoding other polypeptides or proteins.

Tissues, organs or organisms may be contacted with one or more agents of the invention and nucleic acid based vectors, simultaneously or sequentially, at a single time point or at multiple time points. One of ordinary skill in the art will appreciate that the manner and timing of agent administration will be influenced by the duration and degree of inhibition of the agents, pharmaceutical properties of the agent, and underlying disease condition of the affected tissue, organ or organism.

A method to inhibit or treat a condition associated with increased ENaC levels or increased ENaC activity is provided. The method includes contacting a mammal at risk of or having the condition with an effective amount of an agent that

inhibits or decreases transcription of one or more ENaC subunit genes and/or alters the level, amount or activity of a molecule that alters transcription of one or more ENaC subunit genes, and enhances the efficacy of gene therapy vectors.

Also provided is a method to inhibit or treat a condition associated with increased ENaC levels or increased ENaC activity. The method includes contacting a mammal at risk of or having the condition with an effective amount of an agent that inhibits or decreases transcription of one or more ENaC subunit genes and/or alters the level, amount or activity of a molecule that alters transcription of one or more ENaC subunit genes, wherein the agent is a proteasome modulating agent, and wherein the agent is not a gene or gene product encoded by the genome of the mammal, the complement of the gene, or a portion of the gene or its complement.

Further provided is a method to inhibit or treat a condition associated with increased ENaC levels or increased ENaC activity, in which a mammal at risk of or having the condition is contacted with an effective amount of an agent that inhibits or decreases transcription of the α, β, and γ subunits of ENaC or alters the level, amount or activity of a molecule that alters transcription of the α, β, and γ subunits of ENaC.

The invention also provides a method to inhibit or treat a condition associated with increased ENaC levels or increased ENaC activity, which method includes contacting a mammal at risk of or having the condition with an effective amount of an agent that inhibits or decreases transcription of one or more ENaC subunit genes and/or alters the level, amount or activity of a molecule that alters transcription of one or more ENaC subunit genes, and enhances transduction of viruses which infect mammalian cells.

Brief Description of the Figures

Figures 1A-E. Luciferase activity in HeLa cells transfected with rAAV FLAG-Luc in the presence or absence of various agents. HeLa cells were contacted with 100 ppc AAV FLAG-Luc for 2 hours, and cells were harvested 48 hours later. N=3, average $\pm$ standard deviation.

Figure 2. *In vivo* enhancement of rAAV transduction with Doxil. Male Balb/c mice intravenously administered Doxil were endotracheally instilled with 1 x 10^{11} DRP AAV2FLAG-Luc (01:004).

Figure 3. The effects of proteasome inhibitors LLnL and Doxorubicin (Dox) on AV2Luc and AV2/5Luc transduction of immortalized human airway cell lines IB3 (panel A) and A549 (panel B) were evaluated. Proteasome-modulating agents were co-administered with each rAAV vector (MOI of 500 particles per cell) at the time of infection and transduction was evaluated 24 hours later. Various concentrations of each chemical were evaluated as indicated in each graph. Data represent the mean (+/-SEM) relative luciferase activity experiment (N=4).

Figure 4. Dox and LLnL provide additive induction of rAV2 transduction. Hela cells (left panel) and A549 cells (right panel) were infected with rAAV (MOI 500 particles/cell) in the presence of the indicated drug combinations and the expressed transgene was assessed at 24 hours post-infection (Mean +/-SEM, N = 4). Fold induction relative to vehicle-treated rAAV-infected cells is indicated above each bar.

Figure 5. Combined administration of proteasome-modulating agents can synergistically induce rAAV transduction from the apical surface of polarized human airway epithelia. (A) $1x10^9$ particles of AV2Luc were applied to the apical surface of polarized human airway epithelia cultures in the absence and presence of various combinations of LLnL (40 μM) and/or Dox (5 μM). Luciferase expression was assayed at 3 and 17 days post-infection. (B-E) Similar results were observed following apical infection with a self complementary (2.3 kb) scAV2eGFP vector at 15 days post-infection. (F) Combined administration of LLnL and Dox augments dual vector heterodimer-mediated delivery of a trans-spliced LacZ gene product. 10^{10} particles of AV2LacZdonor (indicated by D) and/or AV2LacZacceptor (indicated by A) were used to infect each transwell of the polarized airway epithelia in the presence or absence of co-administered LLnL (40 μM) and Dox (5 μM). β-galactosidase activity was evaluated at 15 days post-infection. Data represents the mean (+/-SEM) relative luciferase or β-galactosidase activity (per 1/10 sample) for 3 independent experiments.

Figure 6. *In vivo* gene transfer to the mouse lung. AV2 and AV2/5 luciferase vectors were used to evaluate the ability of proteasome-modulating agents to induce transduction. Results depict the mean (+/-SEM) luciferase expression from (N = 5) mouse lungs at 14 days post-infection for each condition.

Figure 7. Complementation of CFTR chloride transport abnormalities in CF airway epithelia using combined CFTR rAAV and proteasome modulation. Results depict the mean +/-SEM (N=9) delta Isc response to IBMX/forskolin in CF airway epithelia treated under the indicated conditions marked on the x-axis. The response from Non-CF untreated controls (marked Normal) is given as a reference for fully functional CFTR while all other treatment groups were CF epithelia. Assays were performed at 15 days post-infection.

Figure 8. Expression of transgene-derived and endogenous CFTR mRNA in CF airway epithelia. (A) RS-PCR was used to measure the relative level of rAAV and endogenous CFTR mRNA in all CF airway epithelial samples analyzed in Figure 7. Values represent the mean +/-SEM (N = 9) relative copies of CFTR mRNA. (B) The relative ratio of transgene-derived to endogenous CFTR mRNA was calculated for each sample individually and plotted as an index of the relative level of correction. Values represent the mean +/-SEM (N = 9). A relative ratio of 1 reflects approximately equivalent levels of transgene-derived and endogenous CFTR message.

Figure 9. Quantification of vector DNA following rAAV infection of CF airway epithelia. The total DNA fraction (nuclear and cytoplasmic) remaining following mRNA isolation was quantified by TaqMan PCR for the number of vector genomes for the indicated conditions. Samples are identical to those analyzed in Figures 7 and 8. (A) Values represent the mean +/-SEM (N = 9) relative copies of rAAV CFTR vectors genomes for each sample. (B) The ratio of vector-derived CFTR mRNA to vector DNA was calculated for each individual sample as an index of vector genome transcriptional activity. Higher ratios represent a greater level of transcription per vector genomes for a given condition. Values represent the mean +/-SEM (N = 9).

Figure 10. Proteasome modulation inhibits the function of amiloride-sensitive sodium channels in polarized CF airway epithelia. (A) CF airway epithelial were infected with the indicated viral vectors in the presence or absence of applied LLnL/Dox at the time of infection. Results depict the mean +/- SEM (N = 9) of amiloride-sensitive sodium current in CF airway epithelial cells for each of the indicated treatments. When compared to results in Figures 7 and 8A, the reduction in ENaC activity by LLnL/Dox is independent of the level of CFTR functional correction. (B) CF epithelia were treated with or without LLnL/Dox for 16 hours and total mRNA was prepared at 15 days post-treatment. The abundance of various ENaC subunit mRNAs was measured by Quantitative RT-PCR and the ratios of different ENaC subunits to the level of β-actin mRNA were calculated. The results represent the mean+/-SEM (N = 12 for vehicle group; N = 9 for proteasome modulation group). (C) Kinetics of doxorubicin inhibition of the amiloride-sensitive sodium channel in CF epithelia. Amiloride-sensitive Isc in polarized CuFi cells was measured at 1 day, 3 days, 1 week and 2 weeks after treatment with doxorubicin and compared untreated groups. Results depict the mean +/- SEM of amiloride-sensitive sodium current for each of the indicated treatments (N = 3 for each group).

Figure 11. Doxorubicin treatment increases CpG methylation of the γ-ENaC gene promoter. (A) A549 cells were treated by 40 μM doxorubicin and mRNA was extracted at the indicated time points for Real-Time TaqMan RT-PCR of γ-ENaC subunit mRNA. The ratio of γ-ENaC subunit to β-actin mRNA levels was calculated for each sample. Results represent the mean+/-SEM (N = 3). (B) Schematic diagram of the γ-ENaC gene promoter. The transcription start site is labeled as +1; the position of the CpG island studied herein is shown in black rectangle; the position of restriction enzymes used to study CpG methylation are shown as vertical lines; and the position of primers used in the methylation sensitive PCR analysis are shown by arrows at –3449 and –3139 bp. (C) Results from methylation-sensitive PCR analysis of the –3449 to –3139 bp region of the γ-ENaC gene promoter. MboI digestion of genomic DNA prior to PCR analysis (Lane 9) served as a positive control and gave rise to two PCR products (more than one product is likely due to the GC rich

content of the PCR fragment). When no DNA is added as template (Lane 10), no PCR product is seen. Co-digestion of Dox treated genomic DNA samples with MboI/HpaII (Lane 2-4) gave rise to similar PCR products as seen in the positive control (lane 9), indicating the HpaII sites are protected from digestion by methylation. The extent of protection from HpaII was significantly less in cells not treated with Dox (Lane 1). In contrast, all samples regardless of Dox treatment failed to give PCR production following MboI digestion since this enzyme is not methylation sensitive (Lanes 5-8).

Figure 12. Doxorubicin plays a major role in inhibiting the amiloride-sensitive sodium channel. 40 μM LLnL alone, 5 μM doxorubicin alone, and a combination of these two chemicals, were used to treat the polarized CF airway epithelia from both apical and basal lateral sides. Two weeks later, amiloride-sensitive Isc were measured and compared to the non-treated cells. Results depict the mean +/- SEM of amiloride-sensitive sodium current in CF airway epithelial cells for each of the indicated treatments (N=3 for each group).

Figure 13. Nasal *trans* epithelial potential differences (PD) obtained from 5 week old BLJ6 mice. A) A continuous tracing beginning in 1) Herpes phosphate buffered Ringer's (HPBR), 2) HPBR + 100 μM amiloride, 3) Cl-free HPBR + 100 μM amiloride + 10 μM forskolin. B) A summary of the delta mV change following each buffer switch marked by arrows. C) Absolute mV nasal PD. Values are the mean (+/- SEM) for 5 independent mice.

Figure 14. Screening for anthracycline proteosome modulators. A) Graph of luciferase activity versus concentration of tested agent. B) Fold change in luciferase activity for various treatments.

Figure 15. *In vivo* results for anthracycline proteosome modulators.

Detailed Description of the Invention

Definitions

Agents that "alter ENaC activity" or "inhibit or decrease the level or amount of ENaC" as used herein include but are not limited to agents that inhibit ENaC

activity of a cell, population of cells, tissue, or organ. For example, ENaC activity may be inhibited by inhibiting transcription of one or more ENaC subunit genes, altering the level, amount or activity of a molecule that alters ENaC transcription, altering ENaC RNA stability, and/or altering the trafficking and processing of molecules, for instance, molecules of non-viral origin, through intracellular compartments, including without limitation proteasomes, endosomes, and trans-golgi, and/or through the cytosol, e.g., via cytoskeletal components such as microtubules or microfilaments. One of ordinary skill in the art will recognize that altering ENaC activity may include, for example, decreasing ENaC transcription via direct interaction with the promoter of one or more ENaC subunits, such as methylation of ENaC sequences, or may include affecting the binding of a negatively regulating protein to at least one of the ENaC subunit promoter sequences, e.g., a repressor of ENaC, or alternatively inhibiting binding of a positively regulating transcription factor, e.g., a transcription factor binding protein which binds to one or more of the ENaC promoters. In one embodiment, agents that alter ENaC activity or inhibit or decrease the level or amount of ENaC do not include agents that are antagonists of ENaC, bind a cell membrane bound protein, e.g., bind ENaC or the receptor for hepatocyte growth factor, alter post-translational processing of ENaC, and/or are genes of, or gene products encoded by, a mammalian cell, the complement thereof, or a portion thereof, e.g., an antisense oligonucleotide.

"Dual therapeutic activity," "dual therapeutic action," dual therapeutic," "pharmico-gene therapy," or "potentiate" as used herein to refer to agents of the invention refer to certain agents of the invention that are used to both treat primary pathophysiologic effects of a disease and enhance the efficiency of gene therapy vectors to treat the disease.

A "vector" as used herein refers to a macromolecule, e.g., a polynucleotide, or association of macromolecules that comprises or associates with a polynucleotide, and which can be used to mediate delivery of the polynucleotide to a cell, either *in vitro* or *in vivo*. For instance, a vector may comprise a polynucleotide sequence of recombinant origin. Illustrative vectors include, for

example, plasmids, viral vectors, liposomes and other gene delivery vehicles. The polynucleotide to be delivered, sometimes referred to as a "target polynucleotide" or "transgene," may comprise a coding sequence of interest in gene therapy (such as a gene encoding a protein of therapeutic or interest), a coding sequence of interest in vaccine development (such as a polynucleotide expressing a protein, polypeptide or peptide suitable for eliciting an immune response in a mammal), and/or a selectable or detectable marker.

"AAV" is adeno-associated virus, and may be used to refer to the naturally occurring wild-type virus itself or derivatives thereof. The term covers all subtypes, serotypes and pseudotypes, and both naturally occurring and recombinant forms, except where required otherwise. As used herein, the term "serotype" refers to an AAV which is identified by and distinguished from other AAVs based on capsid protein reactivity with defined antisera, e.g., there are eight serotypes of primate AAVs, AAV-1-AAV-8. For example, serotype AAV2 is used to refer to an AAV which contains capsid proteins encoded from the cap gene of AAV 2 and a genome containing 5' and 3' ITR sequences from the same AAV2 serotype. Pseudotyped AAV as refers to an AAV that contains capsid proteins from one serotype and a viral genome including 5'-3' ITRs of a second serotype. Pseudotyped rAAV would be expected to have cell surface binding properties of the capsid serotype and genetic properties consistent with the ITR serotype. Pseudotyped rAAV are produced using standard techniques described in the art. As used herein, for example, rAAV5 may be used to refer an AAV having both capsid proteins and 5'-3' ITRs from the same serotype or it may refer to an AAV having capsid proteins from serotype 5 and 5'-3' ITRs from a different AAV serotype, e.g., AAV serotype 2. For each example illustrated herein the description of the vector design and production describes the serotype of the capsid and 5'-3' ITR sequences. The abbreviation "rAAV" refers to recombinant adeno-associated virus, also referred to as a recombinant AAV vector (or "rAAV vector").

"Transduction," "transfection," "transformation" or "transducing" as used herein, are terms referring to a process for the introduction of an exogenous polynucleotide, e.g., a transgene in rAAV vector, into a host cell leading to expression of the polynucleotide, e.g., the transgene in the cell, and includes the use

of recombinant virus to introduce the exogenous polynucleotide to the host cell, e.g., viral-mediated transfection is generally referred to as transduction. For example, for AAV the process includes 1) endocytosis of the AAV after it has bound to a cell surface receptor, 2) escape from endosomes or other intracellular compartments in the cytosol of a cell, 3) trafficking of the viral particle or viral genome to the nucleus, 4) uncoating of the virus particles, and generation of expressible double stranded AAV genome forms, including circular intermediates. The rAAV expressible double stranded form may persist as a nuclear episome or optionally may integrate into the host genome. Transduction, transfection or transformation of a polynucleotide in a cell can be determined by methods well known to the art including, but not limited to, protein expression (including steady state levels), e.g., by ELISA, flow cytometry and Western blot, measurement of DNA and RNA by hybridization assays, e.g., Northern blots, Southern blots and gel shift mobility assays. Methods used for the introduction of the exogenous polynucleotide include well-known techniques such as chemical-mediated methods, e.g., Ca^{2+} mediated methods and lipofection, viral infection, and electroporation, as well as non-viral gene delivery techniques. The introduced polynucleotide may be stably or transiently maintained in the host cell.

"Increased transduction or transduction frequency," "altered transduction or transduction frequency," "enhanced transduction or transduction frequency," "increased transfection frequency," "altered transfection frequency," "enhanced transduction frequency," "increased transformation frequency," "altered transformation frequency," "enhanced transformation frequency," refer to an increase in one or more of the activities described above in a treated cell relative to an untreated cell. Agents of the invention which increase transduction, transfection or transformation efficiency may be determined by measuring the effect on one or more of the transduction activities, which may include measuring expression of the transgene, measuring the function of the transgene, or determining the number of vector molecules necessary to yield the same transgene effect compared to host cells not treated with the agents.

"Proteasome modulator" refers to an agent or class of agents which interact with, bind to, or alter the function of, and/or alter the trafficking or location of the proteasome. Proteasome modulators may have other cellular functions as described in the art, e.g., such as doxyrubicin, which is an antibiotic.

"Gene delivery" refers to the introduction of an exogenous polynucleotide into a cell for gene transfer, and may encompass targeting, binding, uptake, transport, localization, replicon integration and expression.

"Gene transfer" refers to the introduction of an exogenous polynucleotide into a cell which may encompass targeting, binding, uptake, transport, localization and replicon integration, but is distinct from and does not imply subsequent expression of the gene.

"Gene expression" or "expression" refers to the process of gene transcription, translation, and post-translational modification.

A "detectable marker gene" is a gene that allows cells carrying the gene to be specifically detected (e.g., distinguished from cells which do not carry the marker gene). A large variety of such marker genes are known in the art.

A "selectable marker gene" is a gene that allows cells carrying the gene to be specifically selected for or against, in the presence of a corresponding selective agent. By way of illustration, an antibiotic resistance gene can be used as a positive selectable marker gene that allows a host cell to be positively selected for in the presence of the corresponding antibiotic. A variety of positive and negative selectable markers are known in the art, some of which are described below.

A "viral vector" as used herein refers to a viral vector comprising a polynucleotide sequence of recombinant origin, typically a sequence of interest for the genetic transformation of a cell. The term viral vector encompasses both vector particles and vector plasmids.

A "viral vector vaccine" refers to a viral vector comprising a polynucleotide sequence not of viral origin (i.e., a polynucleotide heterologous to that virus), that encodes a peptide, polypeptide, or protein capable of eliciting an immune response in a host contacted with the vector. Expression of the polynucleotide may result in

generation of a neutralizing antibody response and/or a cell mediated response, e.g., a cytotoxic T cell response.

An "infectious" virus or viral particle is one that comprises a polynucleotide component which it is capable of delivering into a cell for which the viral species is trophic. The term does not necessarily imply any replication capacity of the virus. A "replication-competent" virus (e.g., a replication-competent AAV, sometimes abbreviated as "RCA") refers to a phenotypically wild-type virus that is infectious, and is also capable of being replicated in an infected cell (i.e., in the presence of a helper virus or helper virus functions).

The term "polynucleotide" refers to a polymeric form of nucleotides of any length, including deoxyribonucleotides or ribonucleotides, or analogs thereof. A polynucleotide may comprise modified nucleotides, such as methylated or capped nucleotides and nucleotide analogs, and may be interrupted by non-nucleotide components. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The term polynucleotide, as used herein, refers interchangeably to double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

A "transcriptional regulatory sequence" or "TRS," as used herein, refers to a genomic region that controls the transcription of a gene or coding sequence to which it is operably linked. Transcriptional regulatory sequences of use in the present invention generally include at least one transcriptional promoter and may also include one or more enhancers and/or terminators of transcription.

"Operably linked" refers to an arrangement of two or more components, wherein the components so described are in a relationship permitting them to function in a coordinated manner. By way of illustration, a transcriptional regulatory sequence or a promoter is operably linked to a coding sequence if the TRS or promoter promotes transcription of the coding sequence. An operably

linked TRS is generally joined in cis with the coding sequence, but it is not necessarily directly adjacent to it.

"Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide). Similarly, a TRS or promoter that is removed from its native coding sequence and operably linked to a different coding sequence is a heterologous TRS or promoter.

"Packaging" as used herein refers to a series of subcellular events that results in the assembly and encapsidation of a viral vector. Thus, when a suitable vector is introduced into a packaging cell line under appropriate conditions, it can be assembled into a viral particle. Functions associated with packaging of viral vectors are described in the art.

A "terminator" refers to a polynucleotide sequence that tends to diminish or prevent read-through transcription (i.e., it diminishes or prevent transcription originating on one side of the terminator from continuing through to the other side of the terminator). The degree to which transcription is disrupted is typically a function of the base sequence and/or the length of the terminator sequence. In particular, as is well known in numerous molecular biological systems, particular DNA sequences, generally referred to as "transcriptional termination sequences" are specific sequences that tend to disrupt read-through transcription by RNA polymerase, presumably by causing the RNA polymerase molecule to stop and/or disengage from the DNA being transcribed. Typical example of such sequence-specific terminators include polyadenylation ("polyA") sequences, e.g., SV40 polyA. In addition to or in place of such sequence-specific terminators, insertions of relatively long DNA sequences between a promoter and a coding region also tend to disrupt transcription of the coding region, generally in proportion to the length of the intervening sequence. This effect presumably arises because there is always some tendency for an RNA polymerase molecule to become disengaged from the DNA being transcribed, and increasing the length of the sequence to be traversed

before reaching the coding region would generally increase the likelihood that disengagement would occur before transcription of the coding region was completed or possibly even initiated. Terminators may thus prevent transcription from only one direction ("uni-directional" terminators) or from both directions ("bi-directional" terminators), and may be comprised of sequence-specific termination sequences or sequence-non-specific terminators or both. A variety of such terminator sequences are known in the art; and illustrative uses of such sequences within the context of the present invention are provided below.

"Host cells," "cell lines," "cell cultures," "packaging cell line" and other such terms denote higher eukaryotic cells, preferably mammalian cells, most preferably human cells, useful in the present invention. These cells can be used as recipients for recombinant vectors, viruses or other transfer polynucleotides, and include the progeny of the original cell that was transduced. It is understood that the progeny of a single cell may not necessarily be completely identical (in morphology or in genomic complement) to the original parent cell.

A "therapeutic gene," "prophylactic gene," "target polynucleotide," "transgene," "gene of interest" and the like generally refer to a gene or genes to be transferred using a vector. In one embodiment, such genes are located within a viral vector thus can be replicated and encapsidated into viral particles. Target polynucleotides can be used in this invention to generate vectors for a number of different applications. Such polynucleotides include, but are not limited to: (i) polynucleotides encoding proteins useful in other forms of gene therapy to relieve deficiencies caused by missing, defective or sub-optimal levels of a structural protein or enzyme; (ii) polynucleotides that are transcribed into anti-sense molecules; (iii) polynucleotides that are transcribed into decoys that bind transcription or translation factors; (iv) polynucleotides that encode cellular modulators such as cytokines; (v) polynucleotides that can make recipient cells susceptible to specific drugs, such as the herpes virus thymidine kinase gene; and (vi) polynucleotides for cancer therapy, such as E1A tumor suppressor genes or p53 tumor suppressor genes for the treatment of various cancers. To effect expression of the transgene in a recipient host cell, it is preferably operably linked to a promoter,

either its own or a heterologous promoter. A large number of suitable promoters are known in the art, the choice of which depends on the desired level of expression of the target polynucleotide; whether one wants constitutive expression, inducible expression, cell-specific or tissue-specific expression, etc. The vector may also contain a selectable marker.

A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated.

"Recombinant," as applied to a polynucleotide means that the polynucleotide is the product of various combinations of cloning, restriction and/or ligation steps, and other procedures that result in a construct that is distinct from a polynucleotide found in nature. A recombinant virus is a viral particle comprising a recombinant polynucleotide. The terms respectively include replicates of the original polynucleotide construct and progeny of the original virus construct.

A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, including replication, duplication, transcription, splicing, translation, or degradation of the polynucleotide. The regulation may affect the frequency, speed, or specificity of the process, and may be enhancing or inhibitory in nature. Control elements known in the art include, for example, transcriptional regulatory sequences such as promoters and enhancers. A promoter is a DNA region capable under certain conditions of binding RNA polymerase and initiating transcription of a coding region usually located downstream (in the 3' direction) from the promoter. Promoters include AAV promoters, e.g., P5, P19, P40 and AAV ITR promoters, as well as heterologous promoters.

An "expression vector" is a vector comprising a region which encodes a polypeptide of interest, and is used for effecting the expression of the protein in an intended target cell. An expression vector also comprises control elements operatively linked to the encoding region to facilitate expression of the protein in the target. The combination of control elements and a gene or genes to which they are operably linked for expression is sometimes referred to as an "expression

cassette," a large number of which are known and available in the art or can be readily constructed from components that are available in the art.

"Genetic alteration" refers to a process wherein a genetic element is introduced into a cell other than by mitosis or meiosis. The element may be heterologous to the cell, or it may be an additional copy or improved version of an element already present in the cell. Genetic alteration may be effected, for example, by transfecting a cell with a recombinant plasmid or other polynucleotide through any process known in the art, such as electroporation, calcium phosphate precipitation, or contacting with a polynucleotide-liposome complex. Genetic alteration may also be effected, for example, by transduction or infection with a DNA or RNA virus or viral vector. Preferably, the genetic element is introduced into a chromosome or mini-chromosome in the cell; but any alteration that changes the phenotype and/or genotype of the cell and its progeny is included in this term.

A cell is said to be "stably" altered, transduced or transformed with a genetic sequence if the sequence is available to perform its function during extended culture of the cell *in vitro*. In preferred examples, such a cell is "inheritably" altered in that a genetic alteration is introduced which is also inheritable by progeny of the altered cell.

The terms "polypeptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, acetylation, phosphonylation, lipidation, or conjugation with a labeling component. Polypeptides such as "CFTR" and the like, when discussed in the context of gene therapy and compositions therefor, refer to the respective intact polypeptide, or any fragment or genetically engineered derivative thereof, that retains the desired biochemical function of the intact protein. Similarly, references to CFTR, and other such genes for use in gene therapy (typically referred to as "transgenes" to be delivered to a recipient cell), include polynucleotides encoding the intact polypeptide or any fragment or genetically engineered derivative possessing the desired biochemical function.

An "isolated" plasmid, virus, or other substance refers to a preparation of the substance devoid of at least some of the other components that may also be present where the substance or a similar substance naturally occurs or is initially prepared from. Thus, for example, an isolated substance may be prepared by using a purification technique to enrich it from a source mixture. Enrichment can be measured on an absolute basis, such as weight per volume of solution, or it can be measured in relation to a second, potentially interfering substance present in the source mixture. Increasing enrichments of the embodiments of this invention are increasingly more preferred. Thus, for example, a 2-fold enrichment is preferred, 10-fold enrichment is more preferred, 100-fold enrichment is more preferred, 1000-fold enrichment is even more preferred.

"Efficiency" when used in describing viral production, replication or packaging refers to useful properties of the method: in particular, the growth rate and the number of virus particles produced per cell. "High efficiency" production indicates production of at least 100 viral particles per cell; preferably at least about 10,000 and more preferably at least about 100,000 particles per cell, over the course of the culture period specified.

An "individual" or "subject" treated in accordance with this invention refers to vertebrates, particularly members of a mammalian species, and includes but is not limited to domestic animals, sports animals, and primates, including humans.

"Treatment" of an individual or a cell is any type of intervention in an attempt to alter the natural course of the individual or cell at the time the treatment is initiated, e.g., eliciting a prophylactic, curative or other beneficial effect in the individual. For example, treatment of an individual may be undertaken to decrease or limit the pathology caused by any pathological condition, including (but not limited to) an inherited or induced genetic deficiency, infection by a viral, bacterial, or parasitic organism, a neoplastic or aplastic condition, or an immune system dysfunction such as autoimmunity or immunosuppression. Treatment includes (but is not limited to) administration of a composition, such as a pharmaceutical composition, and administration of compatible cells that have been treated with a composition. Treatment may be performed either prophylactically or

therapeutically; that is, either prior or subsequent to the initiation of a pathologic event or contact with an etiologic agent.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, virology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al., 1989; Gait, 1984; Freshney, 1987; the series Methods in Enzymology; Miller and Calos, 1987; Weir et al.; Ausubel et al., 1987; Coligan et al., 1991; Coligan et al., 1995; and Scopes, 1994.

I. Agents Useful in the Methods of the Invention

Agents useful to inhibit, treat or prevent conditions associated with aberrant ENaC levels, amount or activity include but are not limited those which inhibit or decrease the level or amount of ENaC, e.g., agents that alter the trafficking and processing of molecules through intracellular compartments, including without limitation proteasomes, endosomes, and trans-golgi, and/or cytosol e.g., via cytoskeletal components such as microtubules or microfilaments, inhibit transcription of one or more ENaC subunit genes and/or alter the level, amount or activity of a molecule that alters ENaC transcription. Classes of agents useful in the invention include but are not limited to antibiotics, chemotherapeutics, lipid lowering agents, and food additives, as well as proteasome modulators, e.g., such as tripeptidyl aldehydes, agents that inhibit calpains, cathepsins, cysteine proteases, and/or chymotrypsin-like protease activity of proteasomes (Wagner et al., 2002; Young et al., 2000; Seisenberger et al., 2001), and agents that modulate the proteasome and ubiquitin pathways, e.g., agents that bind to proteasomes and/or modulate the activity of proteasomes, ubiquitin, ubiquitin carrier protein, or ubiquitin ligase, but do not substantially alter the activity of the proteasome, e.g., the proteolytic activity of the proteasome or of ubiquitin, ubiquitin carrier protein, or ubiquitin ligase. Examples of these agents thus include without limitation antibiotics, e.g., epoxomicin, lipid lowering drugs, e.g., simvastatin, food additives, e.g., tannic acid, and chemotherapeutics, e.g., cisplatin, anthracyclines such as doxorubicin, epirubicin, daunorubicin and idarubicin, and camptothecin.

Cysteine protease inhibitors within the scope of the invention include the cystatins, e.g., cystatin B or cystatin C, antipain, leupeptin, E-64, E-64c, E-64d, KO2 (Wacher et al., 1998), LLnL, Z-LLL, CBZ-Val-Phe-H, cysteine protease inhibitors such as those disclosed in U.S. Patent Nos. U.S. Patent No. 5,607,831, 5,374,623, 5,639,732, 5,658,906, 5,714,484, 5,560,937, 5,374,623, 5,607,831, 5,723,580, 5,744,339, 5,827,877, 5,852,007, and 5,776,718, JP 10077276, JP 8198870, JP 8081431, JP 7126294, JP 4202170, WO 96/21006 and WO 96/40737 as well as Cdz-Leu-Leu-norvalinal (MG115), carbobenzoxy-isoleucyl-(gamma)-t-butyl-L-glutamyl-L-alanyl-L-leucinal (PSI), N-acetyl-leu-leunorleucinal (ALLN), MLN519 (Millennium Pharmaceuticals), [(1R)-3-methyl-1-[[(2S)-3-phenyl-2-[(pyrazinylcarbonyl)- amino]propanoyl]amino]butyl]boronic acid (PS-341, known generically as "bortezomib;" trade name Velcade; Millennium Pharmaceuticals), Z-Ile-Glu(OtBu)-Ala-Leu-H, SRI6975 (2 acetylpyridine N phenylguanylhydrazonedihydrochloride, ALLM (N-acetyl-Leu-Leu-methional), clasto-lactacystin beta lactone, as well as proteasome inhibitors disclosed in Iqbal et al. (1995) and Lee et al. (2000), the disclosures of which are specifically incorporated by reference herein.

In one embodiment, cysteine protease inhibitors are peptides or analogs thereof. For instance, peptide cysteine protease inhibitors within the scope of the invention comprise 2 to 20, more preferably 3 to 10, and even more preferably 3 to 8, amino acid residues. "Amino acid," comprises the residues of the natural amino acids (e.g. Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Hyl, Hyp, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val) in D or L form, as well as unnatural amino acids (e.g. phosphoserine, phosphothreonine, phosphotyrosine, hydroxyproline, gamma-carboxyglutamate; hippuric acid, octahydroindole-2-carboxylic acid, statine, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid, penicillamine, ornithine, citruline, α-methyl-alanine, para-benzoylphenylalanine, phenylglycine, propargylglycine, sarcosine, nor-leucine, nor-valine, and tert-butylglycine). Peptide analogs are molecules which comprise at least one amino acid in D form and/or an unnatural amino acid, or other moiety which is not a natural amino acid.

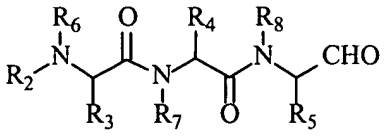
Protease inhibitors include a compound of formula (I): R_1-A-$(B)_n$-C wherein R_1 is an N-terminal amino acid blocking group; each A and B is independently an amino acid; C is an amino acid wherein the terminal carboxy group has been replaced by a formyl (CHO) group; and n is 0, 1, 2, or 3; or a pharmaceutically acceptable salt thereof. In one preferred embodiment, R_1 is $(C_1$-$C_{10})$alkanoyl, acetyl or benzyloxycarbonyl. In another preferred embodiment, each A and B is independently alanine, arginine, glycine, isoleucine, leucine, valine, nor-leucine or nor-valine, and more preferably each A and B is isoleucine. In yet another preferred embodiment, C is alanine, arginine, glycine, isoleucine, leucine, valine, nor-leucine or nor-valine, wherein the terminal carboxy group has been replaced by a formyl (CHO) group, and more preferably, C is nor-leucine or nor-valine, wherein the terminal carboxy group has been replaced by a formyl (CHO) group.

In one embodiment, R_1 is $(C_1$-$C_{10})$alkanoyl. In another embodiment, R_1 is acetyl or benzyloxycarbonyl. In yet a further embodiment, R_1 is $(C_1$-$C_{10})$alkanoyl or benzyloxycarbonyl; A and B are each isoleucine; C is nor-leucine or nor-valine, wherein the terminal carboxy group has been replaced by a formyl (CHO) group; and N is 1. In a further embodiment, C is alanine, arginine, glycine, isoleucine, leucine, valine, nor-leucine or nor-valine, wherein the terminal carboxy group has been replaced by a CHO group, e.g., in one embodiment C is nor-leucine or nor-valine and the terminal carboxy group is replaced by a CHO group. In yet another embodiment, A and B are each independently alanine, arginine, glycine, isoleucine, leucine, valine, nor-leucine or nor-valine, e.g., in one embodiment A and B are each isoleucine.

In a further preferred embodiment, R_1 is $(C_1$-$C_{10})$alkanoyl or benzyloxycarbonyl; A and B are each isoleucine; C is nor-leucine or nor-valine, wherein the terminal carboxy group has been replaced by a formyl (CHO) group; and N is 1.

Also included within the scope of the invention is a compound of formula (II):

(II)



wherein

R_2 is an N-terminal amino acid blocking group;

R_3, R_4, and R_5 are each independently hydrogen, (C_1-C_{10})alkyl, aryl or aryl(C_1-C_{10})alkyl; and

R_6, R_7, and R_8 are each independently hydrogen, (C_1-C_{10})alkyl, aryl or aryl(C_1-C_{10})alkyl; or a pharmaceutically acceptable salt thereof.

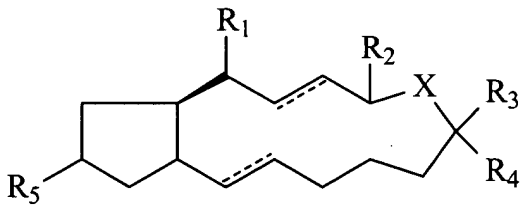
Preferably, R_2 is (C_1-C_{10})alkanoyl, acetyl or benzyloxycarbonyl. Also preferably, R_3 is hydrogen or (C_1-C_{10})alkyl, e.g., 2-methylpropyl. It is preferred that R_4 is hydrogen or (C_1-C_{10})alkyl, e.g., 2-methylpropyl. In another preferred embodiment, R_5 is hydrogen or (C_1-C_{10})alkyl, for example, butyl or propyl. In a further preferred embodiment, R_2 is acetyl or benzyloxycarbonyl; R_3 and R_4 are each 2-methylpropyl; R_5 is butyl or propyl; and R_6, R_7, and R_8 are each independently hydrogen.

R_2 may be (C_1-C_{10})alkanoyl, e.g., acetyl or benzyloxycarbonyl; R_3 may be hydrogen or (C_1-C_{10})alkyl, e.g., 2-methylpropyl. R_5 may be hydrogen or (C_1-C_{10})alkyl, e.g., butyl or propyl. In one embodiment, R_2 is acetyl or benzyloxycarbonyl; R_3 and R_4 are each 2-methylpropyl; R_5 is butyl or propyl; and R_6, R_7, and R_8 are each independently hydrogen.

In one embodiment, R_1 is H, halogen, (C_1-C_{10})alkyl, (C_1-C_{10})alkenyl, (C_1-C_{10})alkynyl, (C_1-C_{10})alkoxy, (C_1-C_{10})alkanoyl, (=O), (=S), OH, SR, CN, NO_2, trifluoromethyl or (C_1-C_{10})alkoxy, wherein any alkyl, alkenyl, alkynyl, alkoxy or alkanoyl may optionally be substituted with one or more halogen, OH, SH, CN, NO_2, trifluoromethyl, NRR or SR, wherein each R is independently H or (C_1-C_{10})alkyl; R_2 is (=O) or (=S); R_3 is H, (C_1-C_{10})alkyl, (C_1-C_{10})alkenyl, (C_1-C_{10})alkynyl, (C_1-C_{10})alkoxy or (C_3-C_8)cycloalkyl, wherein any alkyl, alkenyl, alkynyl, alkoxy or cycloalkyl may optionally be substituted with one or more halogen, OH, CN, NO_2, trifluoromethyl, SR, or NRR, wherein each R is independently H or (C_1-C_{10})alkyl; R_4 is H, (C_1-C_{10})alkyl, (C_1-C_{10})alkenyl, (C_1-C_{10})alkynyl, (C_1-C_{10})alkoxy or (C_3-C_8)cycloalkyl, wherein any alkyl, alkenyl,

alkynyl, alkoxy or cycloalkyl may optionally be substituted with one or more halogen, OH, CN, NO_2, trifluoromethyl, SR, or NRR, wherein each R is independently H or $(C_1$-$C_{10})$alkyl; R_5 is H, halogen, $(C_1$-$C_{10})$alkyl, $(C_1$-$C_{10})$alkenyl, $(C_1$-$C_{10})$alkynyl, $(C_1$-$C_{10})$alkoxy, $(C_1$-$C_{10})$alkanoyl, (=O), (=S), OH, SR, CN, NO_2 or trifluoromethyl, wherein any alkyl, alkenyl, alkynyl, alkoxy or alkanoyl may optionally be substituted with one or more halogen, OH, SH, CN, NO_2, trifluoromethyl, NRR or SR, wherein each R is independently H or $(C_1$-$C_{10})$alkyl; and X is O, S or NR wherein R is H or $(C_1$-$C_{10})$alkyl, or a pharmaceutically acceptable salt thereof.

Another preferred agent useful in the methods of the invention is a compound of formula (III):



wherein

R_1 is H, halogen, $(C_1$-$C_{10})$alkyl, $(C_1$-$C_{10})$alkenyl, $(C_1$-$C_{10})$alkynyl, $(C_1$-$C_{10})$alkoxy, $(C_1$-$C_{10})$alkanoyl, (=O), (=S), OH, SR, CN, NO_2, trifluoromethyl or $(C_1$-$C_{10})$alkoxy, wherein any alkyl, alkenyl, alkynyl, alkoxy or alkanoyl may optionally be substituted with one or more halogen, OH, SH, CN, NO_2, trifluoromethyl, NRR or SR, wherein each R is independently H or $(C_1$-$C_{10})$alkyl;

R_2 is (=O) or (=S);

R_3 is H, $(C_1$-$C_{10})$alkyl, $(C_1$-$C_{10})$alkenyl, $(C_1$-$C_{10})$alkynyl, $(C_1$-$C_{10})$alkoxy or $(C_3$-$C_8)$cycloalkyl, wherein any alkyl, alkenyl, alkynyl, alkoxy or cycloalkyl may optionally be substituted with one or more halogen, OH, CN, NO_2, trifluoromethyl, SR, or NRR, wherein each R is independently H or $(C_1$-$C_{10})$alkyl;

R_4 is H, $(C_1$-$C_{10})$alkyl, $(C_1$-$C_{10})$alkenyl, $(C_1$-$C_{10})$alkynyl, $(C_1$-$C_{10})$alkoxy or $(C_3$-$C_8)$cycloalkyl, wherein any alkyl, alkenyl, alkynyl, alkoxy or cycloalkyl may

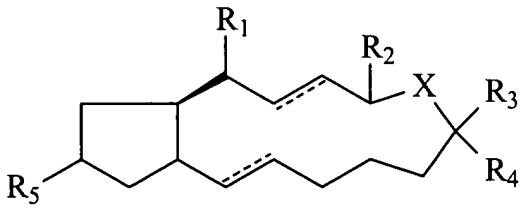
optionally be substituted with one or more halogen, OH, CN, NO_2, trifluoromethyl, SR, or NRR, wherein each R is independently H or (C_1-C_{10})alkyl;

R_5 is H, halogen, (C_1-C_{10})alkyl, (C_1-C_{10})alkenyl, (C_1-C_{10})alkynyl, (C_1-C_{10})alkoxy, (C_1-C_{10})alkanoyl, (=O), (=S), OH, SR, CN, NO_2 or trifluoromethyl, wherein any alkyl, alkenyl, alkynyl, alkoxy or alkanoyl may optionally be substituted with one or more halogen, OH, SH, CN, NO_2, trifluoromethyl, NRR or SR, wherein each R is independently H or (C_1-C_{10})alkyl; and

X is O, S or NR wherein R is H or (C_1-C_{10})alkyl, or a pharmaceutically acceptable salt thereof.

Preferably, R_1 is OH. It is also preferred that R_2 is (=O); R_3 is H or (C_1-C_{10})alkyl, and more preferably R_3 is methyl. Other preferred embodiments include R_4 is H or (C_1-C_{10})alkyl, and more preferably, R_4 is H; R_5 is halogen, CN, NO_2, trifluoromethyl or OH, and more preferably, R_5 is OH. A compound of formula (III) includes X is O or S, preferably O; wherein both ----- are a single bond, wherein one ----- is a double bond, or wherein both ----- are a double bond. In a more preferred embodiment, R_1 is OH, R_2 is (=O), R_3 is methyl, R_4 is H, R_5 is OH, X is O, and both ----- are a double bond.

Yet another agent useful in the methods of the invention is a compound of formula (III):



wherein R_1 is halogen, CN, NO_2, trifluoromethyl or OH. Preferably, R_1 is OH. It is also preferred that R_2 is (=O); R_3 is H or (C_1-C_{10})alkyl, and more preferably R_3 is methyl. Other preferred embodiments include R_4 is H or (C_1-C_{10})alkyl, and more preferably, R_4 is H; R_5 is halogen, CN, NO_2, trifluoromethyl or OH, and more preferably, R_5 is OH. A compound of formula (IV) includes X is O or S, preferably O; wherein both ----- are a single bond, wherein one ----- is a double

bond, or wherein both ----- are a double bond. In a more preferred embodiment, R_1 is OH, R_2 is (=O), R_3 is methyl, R_4 is H, R_5 is OH, X is O, and both ----- are a double bond.

Another agent useful in the methods of the invention includes an agent that inhibits the activation of ubiquitin, the transfer of ubiquitin to the ubiquitin carrier protein, ubiquitin ligase, or a combination thereof. Preferred ubiquitin ligase inhibitors include a compound of formula (IV):

wherein R is hydrogen, an amino acid, or a peptide, wherein the N-terminus amino acid can optionally be protected at the amino group with acetyl, acyl, trifluoroacetyl, or benzyloxycarbonyl;

A is an amino acid or a direct bond;

A_1 is an amino acid; and

R_1 is hydroxy or an amino acid, wherein the C-terminus amino acid can optionally be protected at the carboxy group with (C_1-C_6)alkyl, phenyl, benzyl ester or amide (e.g., $C(=O)NR_2$, wherein each R is independently hydrogen or (C_1-C_6)alkyl);

or a pharmaceutically acceptable salt thereof.

A specific value for R is hydrogen.

A specific value for A is an amino acid. Another specific value for A is Ile, Leu or His. Another specific value for A is Leu or His.

A specific value for A_1 is Ala or Gly. Another specific value for A_1 is Ala.

A specific value for R_1 is hydroxy.

Specifically, the peptide can be a dipeptide (i.e., can comprise 2 amino acids). Specifically, the peptide can be H-Leu-Ala-OH, H-His-Ala-OH, H-Leu-Gly-OH, H-His-Gly-OH, H-Ile-Ala-OH, or H-Ile-Gly-OH. More specifically, the peptide can be H-Leu-Ala-OH or H-His-Ala-OH.

The following definitions apply unless otherwise stated. Alkyl denotes a straight or a branched group, but reference to an individual radical such as "propyl" embraces only the straight chain radical, a branched chain isomer such as

"isopropyl" being specifically referred to. Aryl denotes a phenyl radical or an ortho-fused bicyclic carbocyclic radical having about nine to ten ring atoms in which at least one ring is aromatic.

Suitable N-amino acid blocking groups are known to those skilled in the art (See, for example, T.W. Greene, Protecting Groups In Organic Synthesis; Wiley: New York, 1981, and references cited therein).

Once an agent is identified as useful to inhibit or decrease the level or amount of ENaC, e.g., to inhibit or decrease transcription of ENaC, it may be employed in methods to inhibit reabsorption of salts and water from mucous secretions in a tissue or organ, e.g., in the lung, to hydrate mucous secretions in a tissue or organ, e.g., to increase airway surface liquid volume in the lung, to facilitate mucous clearance in a tissue or organ, to inhibit or treat conditions associated with aberrant ENaC activity, for instance, cystic fibrosis, Liddle syndrome, and pulmonary edema, as well as chronic bronchitis, asthma, and acute lung injury. The identified agents may be administered alone, in combination with other agents, for instance, ENaC antagonists, and/or in combination with gene therapy vectors or vaccine vectors.

II. Introduction of Genetic Material Into Cells

As is described in the art, and illustrated both herein and in the references cited above, genetic material can be introduced into cells (such as mammalian "producer" cells for the production of viral vectors) using any of a variety of means to transform or transduce such cells. By way of illustration, such techniques include, for example, transfection with bacterial plasmids, infection with viral vectors, electroporation, calcium phosphate precipitation, and introduction using any of a variety of lipid-based compositions (a process often referred to as "lipofection"). Methods and compositions for performing these techniques have been described in the art and are widely available.

Selection of suitably altered cells may be conducted by any technique in the art. For example, the polynucleotide sequences used to alter the cell may be introduced simultaneously with or operably linked to one or more detectable or selectable markers as is known in the art. By way of illustration, one can employ a drug-resistance gene as a selectable marker. Drug-resistant cells can then be picked

and grown, and then tested for expression of the desired sequence, i.e., a packaging gene product, or a product of the heterologous polynucleotide, as appropriate. Testing for acquisition, localization and/or maintenance of an introduced polynucleotide can be performed using DNA hybridization-based techniques (such as Southern blotting and other procedures as is known in the art). Testing for expression can be readily performed by Northern analysis of RNA extracted from the genetically altered cells, or by indirect immunofluorescence for the corresponding gene product. Testing and confirmation of packaging capabilities and efficiencies can be obtained by introducing to the cell the remaining functional components of the virus and a helper virus, to test for production of viral particles. Where a cell is inheritably altered with a plurality of polynucleotide constructs, it is generally more convenient (though not essential) to introduce them to the cell separately, and validate each step seriatim. References describing such techniques include those cited herein.

III. Uses of Viral Vectors

Viral vectors can be used for administration to an individual for purposes of gene or vaccine therapy. Suitable diseases for gene or vaccine therapy include but are not limited to those induced by viral, bacterial, or parasitic infections, various malignancies and hyperproliferative conditions, autoimmune conditions, and congenital deficiencies.

Gene or vaccine therapy can be conducted to enhance the level of expression of a particular protein either within or secreted by the cell. Vectors of this invention may be used to genetically alter cells either for gene marking, replacement of a missing or defective gene, or insertion of a therapeutic gene. Alternatively, a polynucleotide may be provided to the cell that decreases the level of expression. This may be used for the suppression of an undesirable phenotype, such as the product of a gene amplified or overexpressed during the course of a malignancy, or a gene introduced or overexpressed during the course of a microbial infection. Expression levels may be decreased by supplying a therapeutic polynucleotide comprising a sequence capable, for example, of forming a stable hybrid with either the target gene or RNA transcript (antisense therapy), capable of acting as a

ribozyme to cleave the relevant mRNA or capable of acting as a decoy for a product of the target gene.

The introduction of viral vectors by the methods of the present invention may involve use of any number of delivery techniques (both surgical and non-surgical) which are available and well known in the art. Such delivery techniques, for example, include vascular catheterization, cannulization, injection, inhalation, inunction, topical, oral, percutaneous, intra-arterial, intravenous, and/or intraperitoneal administrations. Vectors can also be introduced by way of bioprostheses, including, by way of illustration, vascular grafts (PTFE and dacron), heart valves, intravascular stents, intravascular paving as well as other non-vascular prostheses. General techniques regarding delivery, frequency, composition and dosage ranges of vector solutions are within the skill of the art.

In particular, for delivery of a vector of the invention to a tissue, any physical or biological method that will introduce the vector to a host animal can be employed. Vector means both a bare recombinant vector and vector DNA packaged into viral coat proteins, as is well known for virus administration. Simply dissolving a virus vector in phosphate buffered saline has been demonstrated to be sufficient to provide a vehicle useful for muscle tissue expression, and there are no known restrictions on the carriers or other components that can be coadministered with the vector (although compositions that degrade DNA should be avoided in the normal manner with vectors). Pharmaceutical compositions can be prepared as injectable formulations or as topical formulations to be delivered to the muscles by transdermal transport. Numerous formulations for both intramuscular injection and transdermal transport have been previously developed and can be used in the practice of the invention. The vectors can be used with any pharmaceutically acceptable carrier for ease of administration and handling.

For purposes of intramuscular injection, solutions in an adjuvant such as sesame or peanut oil or in aqueous propylene glycol can be employed, as well as sterile aqueous solutions. Such aqueous solutions can be buffered, if desired, and the liquid diluent first rendered isotonic with saline or glucose. Solutions of the viral vector as a free acid (DNA contains acidic phosphate groups) or a pharmacologically acceptable salt can be prepared in water suitably mixed with a

surfactant such as hydroxypropylcellulose. A dispersion of viral particles can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In this connection, the sterile aqueous media employed are all readily obtainable by standard techniques well-known to those skilled in the art.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of a dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the viral vector in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying technique which yield a powder of the active

ingredient plus any additional desired ingredient from the previously sterile-filtered solution thereof.

For purposes of topical administration, dilute sterile, aqueous solutions (usually in about 0.1% to 5% concentration), otherwise similar to the above parenteral solutions, are prepared in containers suitable for incorporation into a transdermal patch, and can include known carriers, such as pharmaceutical grade dimethylsulfoxide (DMSO).

Of particular interest is the correction of the genetic defect of cystic fibrosis, by supplying a properly functioning cystic fibrosis transmembrane conductance regulator (CFTR) to the airway epithelium. Thus, viral vectors encoding native CFTR protein, and mutants and fragments thereof, are all embodiments of this invention.

Compositions of this invention may be used *in vivo* as well as *ex vivo*. *In vivo* gene therapy comprises administering the vectors of this invention directly to a subject. Pharmaceutical compositions can be supplied as liquid solutions or suspensions, as emulsions, or as solid forms suitable for dissolution or suspension in liquid prior to use. For administration into the respiratory tract, a preferred mode of administration is by aerosol, using a composition that provides either a solid or liquid aerosol when used with an appropriate aerosolubilizer device. Another preferred mode of administration into the respiratory tract is using a flexible fiberoptic bronchoscope to instill the vectors. Typically, the viral vectors are in a pharmaceutically suitable pyrogen-free buffer such as Ringer's balanced salt solution (pH 7.4). Although not required, pharmaceutical compositions may optionally be supplied in unit dosage form suitable for administration of a precise amount.

An effective amount of virus is administered, depending on the objectives of treatment. An effective amount may be given in single or divided doses. Where a low percentage of transduction can cure a genetic deficiency, then the objective of treatment is generally to meet or exceed this level of transduction. In some instances, this level of transduction can be achieved by transduction of only about 1 to 5% of the target cells, but is more typically 20% of the cells of the desired tissue type, usually at least about 50%, preferably at least about 80%, more preferably at

least about 95%, and even more preferably at least about 99% of the cells of the desired tissue type. As a guide, the number of vector particles present in a single dose given by bronchoscopy will generally be at least about 1×10^8, and is more typically 5×10^8, 1×10^{10}, and on some occasions 1×10^{11} particles, including both DNAse-resistant and DNAse-susceptible particles. In terms of DNAse-resistant particles, the dose will generally be between 1×10^6 and 1×10^{14} particles, more generally between about 1×10^8 and 1×10^{12} particles. The treatment can be repeated as often as every two or three weeks, as required, although treatment once in 180 days may be sufficient.

To confirm the presence of the desired DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence of a polypeptide expressed from a gene present in the vector, e.g., by immunological means (immunoprecipitations, immunoaffinity columns, ELISAs and Western blots) or by any other assay useful to identify the presence and/or expression of a particular nucleic acid molecule falling within the scope of the invention.

To detect and quantitate RNA produced from introduced DNA segments, RT-PCR may be employed. In this application of PCR, it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique demonstrates the presence of an RNA species and gives information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and only demonstrate the presence or absence of an RNA species.

While Southern blotting and PCR may be used to detect the DNA segment in question, they do not provide information as to whether the DNA segment is being expressed. Expression may be evaluated by specifically identifying the

polypeptide products of the introduced DNA sequences or evaluating the phenotypic changes brought about by the expression of the introduced DNA segment in the host cell.

Thus, the effectiveness of the genetic alteration can be monitored by several criteria. Samples removed by biopsy or surgical excision may be analyzed by in situ hybridization, PCR amplification using vector-specific probes, RNAse protection, immunohistology, or immunofluorescent cell counting. When the vector is administered by bronchoscopy, lung function tests may be performed, and bronchial lavage may be assessed for the presence of inflammatory cytokines. The treated subject may also be monitored for clinical features, and to determine whether the cells express the function intended to be conveyed by the therapeutic polynucleotide.

The decision of whether to use *in vivo* or ex vivo therapy, and the selection of a particular composition, dose, and route of administration will depend on a number of different factors, including but not limited to features of the condition and the subject being treated. The assessment of such features and the design of an appropriate therapeutic regimen is ultimately the responsibility of the prescribing physician.

The foregoing description provides, inter alia, methods for generating high titer preparations of recombinant viral vectors that are substantially free of helper virus (e.g., adenovirus) and cellular proteins. It is understood that variations may be applied to these methods by those of skill in this art without departing from the spirit of this invention.

IV. Dosages, Formulations and Routes of Administration of the Agents of the Invention

Administration of the agents identified in accordance with the present invention may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the agents of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses. Both local and systemic administration is contemplated. When the agents of the invention are

employed for prophylactic purposes, agents of the invention are amenable to chronic use, preferably by systemic administration.

The agents of the invention, including a compound of formula (I), (II), (III), or (IV) including their salts, are preferably administered at dosages of about 0.01 μM to about 1 mM, more preferably about 0.1 μM to about 40 μM, and even more preferably, about 1 μM to 40 μM, although other dosages may provide a beneficial effect. For example, preferred dosages of LLnL include about 1 μM to 40 μM.

One or more suitable unit dosage forms comprising the agents of the invention, which, as discussed below, may optionally be formulated for sustained release, can be administered by a variety of routes including oral, or parenteral, including by rectal, transdermal, subcutaneous, intravenous, intramuscular, intraperitoneal, intrathoracic, intrapulmonary and intranasal routes. For example, for administration to the liver, intravenous administration is preferred. For administration to the lung, airway administration is preferred. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the agent with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

When the agents of the invention are prepared for oral administration, they are preferably combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage form. The total active ingredients in such formulations comprise from 0.1 to 99.9% by weight of the formulation. By "pharmaceutically acceptable" it is meant the carrier, diluent, excipient, and/or salt must be compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof. The active ingredient for oral administration may be present as a powder or as granules; as a solution, a suspension or an emulsion; or in achievable base such as a synthetic resin for ingestion of the active ingredients from a chewing gum. The active ingredient may also be presented as a bolus, electuary or paste.

Pharmaceutical formulations containing the agents of the invention can be prepared by procedures known in the art using well known and readily available ingredients. For example, the agent can be formulated with common excipients, diluents, or carriers, and formed into tablets, capsules, suspensions, powders, and the like. Examples of excipients, diluents, and carriers that are suitable for such formulations include the following fillers and extenders such as starch, sugars, mannitol, and silicic derivatives; binding agents such as carboxymethyl cellulose, HPMC and other cellulose derivatives, alginates, gelatin, and polyvinyl-pyrrolidone; moisturizing agents such as glycerol; disintegrating agents such as calcium carbonate and sodium bicarbonate; agents for retarding dissolution such as paraffin; resorption accelerators such as quaternary ammonium compounds; surface active agents such as cetyl alcohol, glycerol monostearate; adsorptive carriers such as kaolin and bentonite; and lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols.

For example, tablets or caplets containing the agents of the invention can include buffering agents such as calcium carbonate, magnesium oxide and magnesium carbonate. Caplets and tablets can also include inactive ingredients such as cellulose, pregelatinized starch, silicon dioxide, hydroxy propyl methyl cellulose, magnesium stearate, microcrystalline cellulose, starch, talc, titanium dioxide, benzoic acid, citric acid, corn starch, mineral oil, polypropylene glycol, sodium phosphate, and zinc stearate, and the like. Hard or soft gelatin capsules containing an agent of the invention can contain inactive ingredients such as gelatin, microcrystalline cellulose, sodium lauryl sulfate, starch, talc, and titanium dioxide, and the like, as well as liquid vehicles such as polyethylene glycols (PEGs) and vegetable oil. Moreover, enteric coated caplets or tablets of an agent of the invention are designed to resist disintegration in the stomach and dissolve in the more neutral to alkaline environment of the duodenum.

The agents of the invention can also be formulated as elixirs or solutions for convenient oral administration or as solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous or intravenous routes.

The pharmaceutical formulations of the agents of the invention can also take the form of an aqueous or anhydrous solution or dispersion, or alternatively the form of an emulsion or suspension.

Thus, the therapeutic agent may be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The active ingredients may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

These formulations can contain pharmaceutically acceptable vehicles and adjuvants which are well known in the prior art. It is possible, for example, to prepare solutions using one or more organic solvent(s) that is/are acceptable from the physiological standpoint, chosen, in addition to water, from solvents such as acetone, ethanol, isopropyl alcohol, glycol ethers such as the products sold under the name "Dowanol", polyglycols and polyethylene glycols, C_1-C_4 alkyl esters of short-chain acids, preferably ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name "Miglyol", isopropyl myristate, animal, mineral and vegetable oils and polysiloxanes.

The compositions according to the invention can also contain thickening agents such as cellulose and/or cellulose derivatives. They can also contain gums such as xanthan, guar or carbo gum or gum arabic, or alternatively polyethylene glycols, bentones and montmorillonites, and the like.

It is possible to add, if necessary, an adjuvant chosen from antioxidants, surfactants, other preservatives, film-forming, keratolytic or comedolytic agents, perfumes and colorings. Also, other active ingredients may be added, whether for the conditions described or some other condition.

For example, among antioxidants, t-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol and its derivatives may

be mentioned. The galenical forms chiefly conditioned for topical application take the form of creams, milks, gels, dispersion or microemulsions, lotions thickened to a greater or lesser extent, impregnated pads, ointments or sticks, or alternatively the form of aerosol formulations in spray or foam form or alternatively in the form of a cake of soap.

Additionally, the agents are well suited to formulation as sustained release dosage forms and the like. The formulations can be so constituted that they release the active ingredient only or preferably in a particular part of the intestinal or respiratory tract, possibly over a period of time. The coatings, envelopes, and protective matrices may be made, for example, from polymeric substances, such as polylactide-glycolates, liposomes, microemulsions, microparticles, nanoparticles, or waxes. These coatings, envelopes, and protective matrices are useful to coat indwelling devices, e.g., stents, catheters, peritoneal dialysis tubing, and the like.

The agents of the invention can be delivered via patches for transdermal administration. See U.S. Patent No. 5,560,922 for examples of patches suitable for transdermal delivery of an agent. Patches for transdermal delivery can comprise a backing layer and a polymer matrix which has dispersed or dissolved therein an agent, along with one or more skin permeation enhancers. The backing layer can be made of any suitable material which is impermeable to the agent. The backing layer serves as a protective cover for the matrix layer and provides also a support function. The backing can be formed so that it is essentially the same size layer as the polymer matrix or it can be of larger dimension so that it can extend beyond the side of the polymer matrix or overlay the side or sides of the polymer matrix and then can extend outwardly in a manner that the surface of the extension of the backing layer can be the base for an adhesive means. Alternatively, the polymer matrix can contain, or be formulated of, an adhesive polymer, such as polyacrylate or acrylate/vinyl acetate copolymer. For long-term applications it might be desirable to use microporous and/or breathable backing laminates, so hydration or maceration of the skin can be minimized.

Examples of materials suitable for making the backing layer are films of high and low density polyethylene, polypropylene, polyurethane, polyvinylchloride, polyesters such as poly(ethylene phthalate), metal foils, metal foil laminates of such

suitable polymer films, and the like. Preferably, the materials used for the backing layer are laminates of such polymer films with a metal foil such as aluminum foil. In such laminates, a polymer film of the laminate will usually be in contact with the adhesive polymer matrix.

The backing layer can be any appropriate thickness which will provide the desired protective and support functions. A suitable thickness will be from about 10 to about 200 microns.

Generally, those polymers used to form the biologically acceptable adhesive polymer layer are those capable of forming shaped bodies, thin walls or coatings through which agents can pass at a controlled rate. Suitable polymers are biologically and pharmaceutically compatible, nonallergenic and insoluble in and compatible with body fluids or tissues with which the device is contacted. The use of soluble polymers is to be avoided since dissolution or erosion of the matrix by skin moisture would affect the release rate of the agents as well as the capability of the dosage unit to remain in place for convenience of removal.

Exemplary materials for fabricating the adhesive polymer layer include polyethylene, polypropylene, polyurethane, ethylene/propylene copolymers, ethylene/ethylacrylate copolymers, ethylene/vinyl acetate copolymers, silicone elastomers, especially the medical-grade polydimethylsiloxanes, neoprene rubber, polyisobutylene, polyacrylates, chlorinated polyethylene, polyvinyl chloride, vinyl chloride-vinyl acetate copolymer, crosslinked polymethacrylate polymers (hydrogel), polyvinylidene chloride, poly(ethylene terephthalate), butyl rubber, epichlorohydrin rubbers, ethylene vinyl alcohol copolymers, ethylene-vinyloxyethanol copolymers; silicone copolymers, for example, polysiloxane-polycarbonate copolymers, polysiloxane-polyethylene oxide copolymers, polysiloxane-polymethacrylate copolymers, polysiloxane-alkylene copolymers (e.g., polysiloxane-ethylene copolymers), polysiloxane-alkylenesilane copolymers (e.g., polysiloxane-ethylenesilane copolymers), and the like; cellulose polymers, for example methyl or ethyl cellulose, hydroxy propyl methyl cellulose, and cellulose esters; polycarbonates; polytetrafluoroethylene; and the like.

Preferably, a biologically acceptable adhesive polymer matrix should be selected from polymers with glass transition temperatures below room temperature.

The polymer may, but need not necessarily, have a degree of crystallinity at room temperature. Cross-linking monomeric units or sites can be incorporated into such polymers. For example, cross-linking monomers can be incorporated into polyacrylate polymers, which provide sites for cross-linking the matrix after dispersing the agent into the polymer. Known cross-linking monomers for polyacrylate polymers include polymethacrylic esters of polyols such as butylene diacrylate and dimethacrylate, trimethylol propane trimethacrylate and the like. Other monomers which provide such sites include allyl acrylate, allyl methacrylate, diallyl maleate and the like.

Preferably, a plasticizer and/or humectant is dispersed within the adhesive polymer matrix. Water-soluble polyols are generally suitable for this purpose. Incorporation of a humectant in the formulation allows the dosage unit to absorb moisture on the surface of skin which in turn helps to reduce skin irritation and to prevent the adhesive polymer layer of the delivery system from failing.

Agents released from a transdermal delivery system must be capable of penetrating each layer of skin. In order to increase the rate of permeation of an agent, a transdermal drug delivery system must be able in particular to increase the permeability of the outermost layer of skin, the stratum corneum, which provides the most resistance to the penetration of molecules. The fabrication of patches for transdermal delivery of agents is well known to the art.

For administration to the upper (nasal) or lower respiratory tract by inhalation, the agents of the invention are conveniently delivered from an insufflator, nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the composition may take the form of a dry powder, for example, a powder mix of the agent and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or,

e.g., gelatine or blister packs from which the powder may be administered with the aid of an inhalator, insufflator or a metered-dose inhaler.

For intra-nasal administration, the agent may be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and the Medihaler (Riker).

The local delivery of the agents of the invention can also be by a variety of techniques which administer the agent at or near the site of disease. Examples of site-specific or targeted local delivery techniques are not intended to be limiting but to be illustrative of the techniques available. Examples include local delivery catheters, such as an infusion or indwelling catheter, e.g., a needle infusion catheter, shunts and stents or other implantable devices, site specific carriers, direct injection, or direct applications.

For topical administration, the agents may be formulated as is known in the art for direct application to a target area. Conventional forms for this purpose include wound dressings, coated bandages or other polymer coverings, ointments, creams, lotions, pastes, jellies, sprays, and aerosols. Ointments and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilizing agents, dispersing agents, suspending agents, thickening agents, or coloring agents. The active ingredients can also be delivered via iontophoresis, e.g., as disclosed in U.S. Patent Nos. 4,140,122; 4,383,529; or 4,051,842. The percent by weight of an agent of the invention present in a topical formulation will depend on various factors, but generally will be from 0.01% to 95% of the total weight of the formulation, and typically 0.1-25% by weight.

Drops, such as eye drops or nose drops, may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents or suspending agents. Liquid sprays are conveniently delivered from pressurized packs. Drops can be delivered via a simple eye dropper-capped bottle, or via a plastic bottle adapted to deliver liquid contents dropwise, via a specially shaped closure.

The agent may further be formulated for topical administration in the mouth or throat. For example, the active ingredients may be formulated as a lozenge further comprising a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the composition in an inert base such as gelatin and glycerin or sucrose and acacia; and mouthwashes comprising the composition of the present invention in a suitable liquid carrier.

The formulations and compositions described herein may also contain other ingredients such as antimicrobial agents, or preservatives. Furthermore, the active ingredients may also be used in combination with other agents, for example, bronchodilators.

The agents of this invention may be administered to a mammal alone or in combination with pharmaceutically acceptable carriers. As noted above, the relative proportions of active ingredient and carrier are determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmaceutical practice.

The dosage of the present agents will vary with the form of administration, the particular compound chosen and the physiological characteristics of the particular patient under treatment. Generally, small dosages will be used initially and, if necessary, will be increased by small increments until the optimum effect under the circumstances is reached.

The invention will be further described by, but is not limited to, the following examples. The following examples illustrating the use of some of the agents of the invention to enhance AAV transduction employed AAV2 and AAV5 serotypes as well as pseudotyped AAV5/2 virus. However, it is contemplated that that agents of the invention are useful for all serotypes and pseudotypes of rAAV vectors.

Example 1

Endosomal Processing Limits AAV Transduction

Based on the finding that basolateral membranes have higher endocytic rates and UV irradiation enhances endosomal uptake and rAAV transduction from the apical membrane, it is possible that endosomal pathways influencing viral uptake

and transport to the nucleus may be limiting from the apical membrane. In contrast, these pathways may be active at maximal levels from the basolateral membrane of airway epithelial cells. To further investigate the importance of endosomal processing, the effect(s) of several chemical agents known to alter endosomal processing was evaluated.

Methods

Initial studies were performed in confluent primary human fibroblasts since dose titrations and toxicity could be quickly assessed. Selected agents were used to treat fibroblast monolayers prior to rAAV infection. rAAV transduction was assessed at 96 hours post-infection by FACS analysis, and the percentage of dead cells was simultaneously assessed by incorporation of propidium iodide.

These agents included nocodazole (Sigma, St. Louis, MO; depolymerizes microtubules and causes lysosomal scattering); vinblastine sulfate (Sigma, St. Louis, MO; depolymerizes microtubules, inhibits endocytosis by blocking intracellular endosomes and lysosomes movement); cytochalasin B (Sigma, St. Louis, MO; depolymerizes microfilaments, i.e., actin, and blocks fusion of endosome with lysosome. Inhibits endocytosis by blocking intracellular endosome and lysosome movement); brefeldin A (BFA, Sigma, St. Louis, MO; reversibly blocks protein transport from the ER to the Golgi. BFA has also been shown to increase endocytosis from the apical but not basolateral membranes, see Prydz et al. (1992)); NH_4Cl (Sigma, St. Louis, MO; lysosomotropic reagent which raises endosomal pH, and has been shown to inhibit canine parvovirus uncoating, see Basak et al.(1992)); chloroquine (Sigma, St. Louis, MO; lysosomotropic reagent which raises endosomal pH and inhibits lysosomal cysteine protease cathepsin B, and has been shown to inhibit canine parvovirus uncoating, see Basak et al. (1992)); and LLnL (N-acetyl-L-Leucinyl-L-leucinal-L-norleucinal; Calbiochem-Novabiochem Corp., La Jolla, CA) and Z-LLL (N-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norvalinal; Calbiochem-Novabiochem Corp., La Jolla, CA), which are tripeptidyl aldehydecysteine protease inhibitors. These tripeptides are structurally related to chloroquine but have different lipid solubility and specificity for cysteine proteases (Seglen, 1983). These molecules decrease endosomal degradation of molecules by

a mechanism different than altering pH. They also have been shown to inhibit 26S ubiquitin and proteasome-dependent proteolytic pathways (Rock et al., 1994).

Results

As previously reported for canine parvovirus (Basake et al., 1992), both NH_4Cl and chloroquine, which raise the endosomal pH, significantly inhibited rAAV transduction. These results support the importance of endosomal pH in facilitating virus release and/or uncoating following infection. Moreover agents such as cytochalasin B, which disrupt microfilament formation, led to a significant decrease in rAAV transduction, suggesting that actin microfilaments likely play some role in rAAV transduction. Further, vinblastine, which facilitates both microtubule depolymerization and decreases endocytosis in MDCK cells, had little effect on rAAV transduction.

Most interestingly, however, treatment with BFA, which disrupts ER to Golgi vesicular transport and has also been shown to increase apical membrane endocytosis in MDCK cells (Prydz et al., 1992), led to a significant enhancement of rAAV transduction. The importance of ER to Golgi vesicular transport is unclear, but given the findings that UV irradiation also enhances membrane endocytosis and BFA has been suggested to do the same, these findings suggested that the rate of membrane endocytosis of receptor bound rAAV may be a limiting step in transduction. Similarly to BFA, two endosomal protease inhibitors (tripeptides LLnL and Z-LLL) both significantly increased rAAV transduction. These tripeptides have been previously used to increase the transfection efficiency of plasmid DNA and are thought to inhibit the lysosomal degradation of DNA (Coonrod et al., 1997).

The data support the hypothesis that endocytosis and endosomal processing is a key rate-limiting step in rAAV transduction. It appears that actin microfilaments, but not microtubules, are important in rAAV transduction and may act by facilitating rAAV transport to the nucleus. Moreover, cytochalasin B efficiently blocks apical but not basolateral infection of the polarized MDCK cells with influenza virus (Gottlieb et al., 1993). These findings indicate that there is a fundamental difference in the process by which endocytic vesicles are formed at the

two surfaces of polarized epithelial cells, and that the integrity and/or the polymerization of actin filaments is required at the apical surface. However, the findings that microtubule depolymerizing agents such as vinblastine did not inhibit rAAV-2 transduction are different than that previously reported for nocodazole inhibition of canine parvovirus (Vihinen-Tanta et al., 1998). Lastly, studies with tripeptide protease inhibitors demonstrated a significant augmentation in rAAV transduction. Such findings suggest that endosomal degradation of virus and/or endosomal release may be an important rate-limiting step in rAAV transduction.

Example 2

Endosomal Processing Inhibitors May Increase rAAV Transduction in Polarized Airway Cells

Materials and Methods

Primary culture of human bronchial epithelia and reagents utilized. Primary human airway epithelial cells were collected by enzymatic digestion of bronchial samples from lung transplants, as previously described (Kondo et al., 1991; Zabner et al., 1996). Isolated primary airway cells were seeded at a density of 5×10^5 cells/cm^2 onto collagen-coated Millicell-HA culture inserts (Millipore Corp., Bedford, MA). Primary cultures were grown at the air-liquid interface for more than 2 weeks, by which time differentiation into a mucociliary epithelium occurs. The culture medium, used to feed only the basolateral side of the cells, contained 49% DMEM, 49% Ham's F12 and 2% Ultraser G (BioSepra, Cedex, France). Dimethyl Sulphoxide (DMSO), camptothecin (Camp), etoposide (Etop), aphidicolin (Aphi), hydroxyurea (HU) and genistein (Geni) were purchased from Sigma (St. Louis, MO). Tripeptidyl aldehyde proteasome inhibitors N-Acetyl-L-Leucyl-L-Leucyl-Norleucine (LLnL) and benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal (Z-LLL) were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA). Ubiquitin ligase (E3) inhibitors were obtained from Bachem Bioscience Inc. (King of Prussia, PA). Anti-AAV capsid monoclonal antibody (Anti-VP1,2 and 3) was purchased from American Research Products (Belmont, MA) and anti-ubiquitin antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Production of recombinant AAV viral stocks. Recombinant AAV was produced by a $CaPO_4$ co-transfection protocol and purified through three rounds of isopycnic cesium chloride ultracentrifugation as described above in Example 1. The proviral plasmid pCisAV.GFP3ori is described in Duan et al. (1998). The proviral plasmid pCisRSV.Alkphos, which encodes the alkaline phosphatase reporter gene under the transcriptional control of the RSV promoter and SV40 poly-adenylation signal, was used to generate AV.Alkphos (Yang et al., 1999). The proviral plasmid pCisRSV.LacZ used for AV.LacZ production was generated by first inserting 3474 bp Not I digested β-galactosidase gene (from pCMVβ, Clontech) into the Not I site of the pRep4 (Invitrogene). The entire β-galactosidase expression cassette, including the RSV promoter, β-galactosidase reporter gene and SV40 polyA signal, was excised by Sal I and subsequently cloned into the pSub201 backbone by blunt end ligation (Samulski et al., 1987). Recombinant viral stocks were heated at 58□C for 60 minutes to inactivate contaminating helper adenovirus. Typical yields were 5×10^5 to 5×10^9 particles/μl based on DNA slot blot hybridization assays against plasmid standards. The level of adenoviral contamination, as based on a second reporter assay (Duan et al., 1997) for the recombinant adenovirus used for propagation (Ad.CMVAlkphos for AV.GFP3ori, and Ad.CMVLacZ for AV.Alkphos, Ad.CMVGFP for AV.LacZ), was less than one functional particle per 1×10^{10} rAAV particles used for infection of 293 cells in the presence of adenovirus. Transfection with Rep/Cap encoding plasmids served as controls for antibody staining of Rep protein. Virus was dialyzed in PBS prior to *in vitro* or *in vivo* infections.

Transduction of polarized airway epithelial cells and primary human fibroblasts. rAAV infection of fully differentiated bronchial cells was performed as described in Duan et al. (1998). For infections from the apical surface of the airway cells, 5 μl rAAV was mixed with 50 μl of culture media and applied directly onto the apical compartment of Millicell inserts (MOI=10,000 particles/cell). During apical infection, the basolateral side of the Millicell was continuously bathed in culture media. Gene transfer to the basal side was performed by inverting Millicell inserts and applying viral vector to the bottom of the supporting filter membrane in

a 50 μl volume for 2 hours. Subsequently, Millicell inserts were returned to the upright position, in the continued presence of the original viral inoculum plus an additional 450 μl of media. For both apical and basolateral infections, rAAV containing media was removed after 24 hours and replaced with either fresh culture media (for the basal side) or exposed to air (for the apical side). To test the effect of different agents on the efficiency of AAV transduction in polarized airway cells, 1 μl of each solution was mixed with AAV prior to infection of airway epithelia. Agents were usually presented during the 24 hours AAV infection period unless indicated otherwise. Most of the agents were dissolved in DMSO except for hydroxyurea (dissolved in phosphate buffered saline), H-Leu-Ala-OH (dissolved in 0.9% glacial acetic acid) and H-His-Ala-OH (dissolved in 50% methanol). The working concentrations of the agents were as follows: 0.1 μM camptothecin, 10 μM etoposide, 5 μg/ml aphidicolin, 40 mM hydroxyurea, 50 μM genistein, 40 μM LLnL and 4 μM Z-LLL. When the ubiquitin ligase (E3) inhibitors (H-Leu-Ala-OH and H-His-Ala-OH) were used, airway cells were pretreated with a combination of both inhibitors at a final concentration of 2 mM for 60 minutes prior to infection, followed by the continued presence of inhibitor (0.2 mM) during the entire 24 hours infection period from the basolateral surface. Studies involving EGTA treatment were performed by transiently treating the apical membrane of polarized airway epithelia with 3 mM EGTA in water for 10 minutes (Duan et al., 1998). Following hypotonic EGTA treatment, cultures were washed twice with culture medium and infected with rAAV in the presence or absence of 40 μM LLnL. Human primary fibroblast cells (P4) were maintained in 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, and 89% DMEM. Infection with AV.GFP3ori was performed with 80% confluent fibroblasts at an MOI of 1000 DNA particles/cell in 2% FBS DMEM for 24 hours.

S^{35} labeling of rAAV. The methionine residue in the capsid protein of rAV.GFP3ori was labeled during the generation of radioactive viral stocks according to a previously published protocol with modifications (Mizukami et al., 1996). Briefly, twenty 150 mm plates of subconfluent 293 cells were infected with Ad.LacZ (5 pfu/cell) for 1 hour followed by calcium phosphate transfection of

pCisAV.GFP3ori (250 μg) and pRepCap (750 μg). Cells were incubated for an additional 10 hours, at which time the medium was changed to 2% FBS Methionine-free DMEM for 45 to 60 minutes. The medium was changed once again to labeling medium containing 15 mCi of S^{35}-methionine per 400 ml of 2% FBS Methionine-free DMEM (final = 1.49 MBq/ml), and cells were pulsed for 1.5 hours at 37°C. Following labeling, L-methionine was added back to a final concentration of 30 mg/L, and cells were incubated for an additional 30 hours at 37°C. Cell lysates were prepared and virus was purified by isopycnic cesium chloride ultracentrifugation as described above. Typical specific activities of labeled virus preparations were 5×10^{-6} cpm/particle, which is slightly higher than the 5.5×10^{-7} cpm/particle specific activity reported by other investigators (Bartlet et al., 1999).

Viral binding/entry assays and in situ localization of viral particles. To assess the binding of rAAV to polarized bronchial epithelia cells, S^{35}-labeled AV.GFP3ori was applied to either the apical or basal surface (MOI=50,000 particles/cell), followed by incubation at 4°C for 60 minutes. Combined binding/entry of rAAV into differentiated airway epithelia was measured under the same conditions, except that the cultures were incubated at 37°C for an additional 2-24 hours before they were harvested. These combined viral binding/entry assays were performed under identical infection conditions to those used for functional studies of rAAV transduction with transgene expression as an endpoint. After washing three times in PBS, cells were lysed in situ by the addition of 5 ml of liquid scintillation cocktail at room temperature for 5 minutes, and the radioactivity was quantitated in a scintillation counter.

To analyze the subcellular localization of the rAAV particles within polarized human bronchial epithelial cells, infection was performed by applying S^{35} labeled virus (MOI=50,000 particles/cell) to either the mucosal or serosal surface. At 2 hours post-infection, transwells were washed with medium three times and fixed in 4% paraformaldehyde overnight prior to cryoprotection and embedding for frozen sectioning. 10 μm frozen sections were overlaid with photoemulsion and developed for 5 weeks according to a previously published protocol (Duan et al., 1998).

Molecular analysis of rAAV viral genomes following infection of polarized airway epithelial cultures. The molecular state of bound and endocytosed virus was assayed at different times following rAAV infection. To examine the amount of virus attached to the cell surface, rAAV infection was performed at 4°C for 1 hour. Following binding, the extent of viral internalization was assessed by continuing incubations in the presence of virus at 37°C for 4-24 hours. Viral DNA was extracted according to a modified Hirt protocol and Southern blots performed with Hybond N+ nylon membrane (Amersham) (Duan et al., 1997). The 1.6 kb single stranded viral DNA, the 2.7 kb double stranded circular intermediate, and the 4.7 kb double stranded replication from viral genome were detected with a transgene EGFP specific probe at 5×10^6 cpm/ml. Blots were washed at a stringency of 0.2 × SSC/0.1%SDS at 55°C for 20 minutes twice. In studies aimed at evaluating viral internalization, virus attached to the cell surface was removed by trypsinization with 1 ml of buffer containing 0.5% trypsin, and 5.3 mM EDTA at 37°C for 10 minutes (500 μl buffer was added to the apical and basolateral compartment of the Millicell inserts), followed by washing with ice-cold PBS twice. Externally bound AAV virus was determined by the intensity of the 1.6 kb viral genome band in Hirt DNA extracted from cells infected at 4°C for 60 minutes. The internalized virus was determined by the intensity of the 1.6 kb viral genome band in Hirt DNA extracted from trypsinized cells after infection at 37°C for 4 and 24 hours. The dynamic changes in the molecular structure of the internalized virus were assayed at 2, 10, 30 and 50 days after virus was removed from culture medium.

Detection of ubiquitinated AAV capsid proteins by immunoprecipitation. To analyze the effect of the proteasome inhibitor on AAV ubiquitination, human primary fibroblasts were lysed at 6 hours post-viral infection in 1X RIPA buffer. Cell lysates were then cleared with 30 μl Protein A-Agarose. The supernatant was incubated with 10 μl of monoclonal anti-VP1, 2, and 3 antibody (Clone B1, ARP) followed by the addition of 30 μl Protein A-Agarose. The pellets were washed 4 times with IX RIPA buffer and resolved on a 10% SDS-PAGE. After transfer to a nitrocellulose filter, blots were probed with a 1:1000 dilution of anti-ubiquitin monoclonal antibody (clone P4D1, Santa Cruz, catalogue #sc-8017), followed by

1:500 HRP-conjugated secondary antibody (BMB). After the final washings, immunoreactivity was visualized using the ECL system (Amersham).

In vivo studies in mice. Animal studies were performed in accordance with the institutional guidelines of the University of Iowa. To determine the effect of the proteasome inhibitor on AAV mediated gene transfer in mouse lung, 6 week-old BALB/c mice were lightly anesthetized using a methoxyflurane chamber. AV.LacZ (5×10^{10} particles) was administered alone or with 400 μM Z-LLL in a 10 μl instillation by nasal aspiration as described by Walters et al. (2000). To prevent unforeseen toxicity of DMSO solvent, the proteasome inhibitor Z-LLL was dissolved in ethanol as a 40 mM stock solution and was included in the viral inoculum at 1% final concentration. Viral infection controls in the absence of Z-LLL also contained a 1% final concentration of ethanol. Since studies in both primary cultured human airway cells and fibroblasts have demonstrated similar enhancement efficiency between 40 μM LLnL and 4 μM Z-LLL, and also due to the poor solubility of LLnL in ethanol (Example 7 employed a low dose of LLnL in DMSO which was administered to the trachea), only Z-LLL was tested in this particular mouse lung study. The animals were euthanized at 2, 10 and 150 days post infection and PBS (10 ml) was instilled into the right ventricle, followed by removal of the lungs and heart as an intact cassette. The trachea was incubated and instilled at 10 cm of water pressure with the following solutions in order: PBS, 0.5% glutaraldehyde, 1 mM $MgCl_2$/PBS, and finally X-gal staining reagent for an overnight incubation at room temperature. The X-gal stained mouse lungs were then post fixed in 10% neutral buffered formalin for 48 hours at room temperature and cryopreserved in serial 10%, 20% and 30% sucrose/PBS solutions. Lungs (N=3 for each condition) were embedded in OCT (optimal cutting temperature; Baxter, Warrendale, PA) and 15 μm serially sections were analyzed for gene transfer by calculating the percentage of positive cells in the airway epithelium. The diameter of the airway was recorded for classification (> 360 μm, 260-350 μm, 160-250 μm, < 150 μm) of results following morphometric analysis. Greater than 150 airway cross-sections were quantified for each experimental condition.

Results

Molecular analysis of rAAV genomes in polarized airway epithelia. Recent studies revealed a lack of AAV-2 receptor, heparin sulfate proteoglycan, and co-receptors, FGFR-1 and $\alpha V\beta 5$ integrin, at the apical surface of differentiated airway epithelia (Duan et al., 1998; Duan et al., 1999; Hughes et al., 1993; Goldman et al., 1999). However, differences in the binding of radioactive virus at the apical and basolateral membranes were only 4-7 fold (basolateral > apical) (Duan et al., 1998). These differences in binding are insufficient to explain the 200-fold variance observed in the polarity of infection (basolateral >> apical) with rAAV-2 (Duan et al., 1998). These findings suggested that viral binding and/or uptake were not the sole limiting factors contributing to inefficient mucosal transduction in airway epithelia. To this end, the molecular state of rAAV DNA at 50 days following apical and basolateral infection of air-liquid interface cultured human bronchial epithelia was evaluated. At this time point, gene expression measured from an EGFP reporter was > 200-fold higher in basolaterally infected cultures (data not shown) (Duan et al., 1998). Hirt DNA from the cultures was evaluated by Southern blot hybridization with ^{32}P-labeled EGFP probes. A significant amount of apically applied rAAV was able to infect airway cells. However, only single stranded viral genomes (ssDNA) were detected at this time point (50 days). This result clearly suggests that rAAV can be endocytosed from the mucosal surface and that the endocytosed viral ssDNA was stably sequestered in some unknown subcellular compartment. In contrast, the majority of basolaterally applied rAAV was converted into double stranded forms that migrated at 2.8 kb and > 12 kb in 1% non-denaturing agarose gels. Consistent with previous reports (Sanlioglu et al., 1999; Duan et al., 1999), subsequent restriction enzyme mapping of Hirt DNA and Southern blots confirmed this 2.8 kb band to be a supercoiled, circular episomal molecule (data not shown). The identity of the > 12 kb band, which is significantly more intense following basolateral infection, is currently unknown but may represent episomal circular concatamers of the AAV genome. Taken together, these results suggest that inefficient molecular conversion of AAV viral DNA to circular genomes represents a significant obstacle for rAAV mediated gene transfer from the apical surface of the airway. Furthermore, circularization, not linear replication

though self-priming, is the predominant pathway for rAAV gene conversion in polarized airway epithelia.

Proteasome modulators dramatically enhance rAAV infection in polarized airway epithelia. Given the fact that rAAV appears to remain latent within some cellular compartment(s) following apical infection in the airway, and that agents that alter the molecular conversion of the viral genome might enhance rAAV transduction in airway epithelia, several agents were tested in this regard, including DNA damaging agents (Alexander et al., 1994), DNA synthesis and topoisomerase inhibitors (Russell et al., 1995), and cellular tyrosine kinases inhibitors (Qing et al., 1997; Man et al., 1998). Application of camptothecin, etoposide, hydroxyurea, and genistein resulted a 10 to 60 fold enhancement in rAAV transduction from the basolateral surface. Interestingly, however, none of these agents facilitated rAAV transduction from the apical surface (data not shown). Since chemicals known to affect intra-nuclear events involved in rAAV transduction in other cell types (Sanlioglu et al., 1999) did not enhance rAAV apical infection in the airway, other agents affecting endocytic processing, such as the ubiquitin-proteasome pathway, were tested. Proteasome systems are known to modulate the intracellular processing of many foreign and endogenous molecules, including viruses such as HIV (Schwartz et al., 1998). Several specific, cell permeable, peptide aldehyde inhibitors of proteasome pathways have recently been discovered (Rock et al., 1994; Fenteany et al., 1995). These inhibitors bind to the active sites of proteolytic enzymes within the proteasome core and reversibly block their function (Rubin et al., 1995). To test whether proteasomes represent an intracellular compartment that sequesters rAAV following infection, the tripeptidyl aldehyde proteasome inhibitor (a cysteine protease inhibitor) N-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL, also called Calpain inhibitor I) was applied to polarized cultures of human bronchial epithelial cells at the time of rAAV infection. Surprisingly, a greater than 200 fold augmentation in transgene expression was obtained at 2 days post infection when 40 μM LLnL was applied to the serosal surface along with rAAV. A similar result was achieved when another ubiquitin-proteasome pathway inhibitor, N-carbobenzoxyl-L-leucinyl-L-leucinyl-L-leucinal (Z-LLL, also called MG132) (Jensen et al., 1995),

was tested (data not shown). However, the most important finding was that these proteasome inhibitors also substantially increased rAAV transduction from the mucosal surface (see below). When compared with other agents, proteasome inhibitors were found to be the most potent enhancers of rAAV transduction in airway epithelium.

Proteasome modulators augment rAAV transduction in airway epithelia in a polarized fashion. Although proteasome modulators appear to significantly increase the efficacy of rAAV transduction from the serosal surface, the route most germane to clinical application of gene delivery in the airway is the mucosal surface. To test the effect of proteasome inhibitors on rAAV transduction from apical membrane, a side-by-side kinetic comparison of rAAV transduction from both mucosal and serosal surfaces of airway epithelia following treatment with LLnL was performed. Co-administration of LLnL and rAAV to the mucosal surface resulted a sustained augmentation in AAV transduction, which peaked at 22 days post-infection. In contrast to mucosal infection, rAAV infection from the serosal surface in the presence of LLnL resulted only in a transient peak in gene expression at 72 hours post-infection, which returned to the levels equivalent to that of the untreated samples by 22 days. These results suggested that the proteasome inhibitor LLnL produces different augmentation profiles when AAV virus is applied to either the apical or the basolateral membranes. To exclude potential effects caused by polarized uptake of LLnL by airway epithelia, different combinations of rAAV and LLnL administration from both apical and basolateral surfaces were tested. Similar augmentation patterns for AAV transduction were achieved, regardless of whether LLnL was applied to the same or opposite surface as rAAV during infections (data not shown).

To determine whether LLnL administration augmented rAAV transduction of particular airway cell types, a rAAV vector encoding the alkaline phosphatase gene (Alkphos) was utilized. Transduced cell types were evaluated by standard histochemical staining for Alkphos to address this question. In the absence of LLnL, rAAV preferentially transduced basal cells at 3 days following serosal application of virus. Consistent with previous findings utilizing AV.GFP3ori virus,

co-administration of LLnL resulted in a dramatic increase in AV.Alkphos transduction. Interestingly, ciliated cell transduction was most significantly increased by treatment with LLnL at the time of rAAV infection. In contrast, basal cells were the least responsive to LLnL treatment. These findings indicated that the mechanisms of LLnL action may have some cell specific components, which differs in polarized (i.e., ciliated) and non-polarized (i.e., basal) cell types.

Optimization of LLnL enhanced rAAV transduction. With the aim of further improving the enhancement in rAAV transduction achieved in the presence of LLnL, several detailed kinetic studies were performed which altered the timing and number of LLnL administrations following rAAV infection. Several important conclusions arose from these studies. First, following basolateral infection, administration of LLnL once every three days increased length of peak transgene expression, despite the fact that by the end of 30 days levels were similar to that of cultures treated once at the time of infection. Second, continual administration of LLnL was toxic to cells and ablated transgene expression by 10 days. Third, re-infection of cultures with rAAV in the presence of LLnL at 7, 10 and 15 days resulted in a similar pattern of augmentation and, as expected, elevated the final level of transgene expression observed at 30 days (only data from the second infection at 15 days are shown). Most notably however, all the cultures infected from the basolateral side produced similar long-term transgene expression levels within 2 to 3 fold of each other, regardless of whether LLnL was administered.

Despite the fact that LLnL administration at the time of the viral infection augmented rAAV transduction from both the apical and basolateral surfaces, the kinetics of this induction were significantly different. Enhancement following basolateral infection was transient, while enhancement following apical infection was long-term. Furthermore, although induction with LLnL from the apical membrane was long-lasting, by 30 days the maximal level of transgene expression was only one eighth of that resulting from basal infection. The application of hypotonic EGTA solution has been shown to increase AAV transduction from the apical surface by 7 to 10 fold (Duan et al., 1998; Walters et al., 2000). Therefore the combined administration of EGTA and LLnL could provide yet a further

increase in rAAV transduction from the apical surface. Interestingly, treatment of airway cultures with EGTA prior to infection with rAAV in the presence of LLnL gave a transient peak in transduction within the first three days, and a significantly increased (200-fold), prolonged level of transgene expression out to 30 days. This prolonged level of transgene expression, while comparable to rAAV infection from the basal surface, was much above the level observed in apically infected epithelia treated with EGTA alone. In summary, these results demonstrate that EGTA and LLnL have synergistic effects on rAAV transduction, allowing for transduction from the apical surface at levels normally only seen following basolateral infection.

Viral binding and internalization are not affected by LLNL treatment. The action of LLnL has been typically attributed to it selective and reversible inhibition of the proteasome system. However, it was important to rule out any possible effect on viral binding and/or endocytosis. As has been found for type 1 herpes simplex virus (Everett et al., 1998), LLnL treatment had no significant effect on 4°C rAAV binding. Similarly, the uptake of S^{35} labeled rAAV for a 2 hour infection period at 37°C was not altered by LLnL treatment. Given these results, LLnL acts at points distal to virus binding and entry. Interestingly, at 24 hours post-infection a very significant decrease in the amount of intracellular radioactivity was observed in epithelia treated with LLnL, regardless of which surface was infected. Given the concordant increase in transgene expression at this time point, LLnL may be accelerating processing and routing of the virus to the nucleus, wherein uncoating and clearance of S^{35} labeled capsid proteins occur. By this mechanism, S^{35} isotope would be diluted into the culture medium and could explain the decrease in cell-associated counts.

LLnL enhances endosomal processing and nuclear trafficking of rAAV. To test the hypothesis that LLnL increases trafficking of rAAV to the nucleus, in situ localization of the S^{35}-labeled rAAV particles following infection from the apical and basolateral surfaces was performed in the presence and absence of LLnL. Since loss of intact radiolabeled capsid proteins occurred at 24 hours post-infection, a 2 hour time point was chosen for this analysis. Using photoemulsion overlay, the subcellular distribution of S^{35}-labeled rAAV particles was evaluated by blinded

morphometric analysis. The majority of viral particles localized to the cytoplasm in the absence of LLnL. This was the case regardless of whether infection was performed from the apical or basolateral surface. In contrast, LLnL treatment substantially changed the intracellular distribution of radiolabeled rAAV particles, resulting in a significant shift to nuclear associated grains. These results substantiated the findings from whole cell counts at 24 hours post-infection, which suggested that LLnL increases viral uncoating and the subsequent loss of S^{35} isotope into the media.

LLnL augment rAAV transduction within a specific time frame after infection. Evidence thus far has suggested that LLnL may act to increase intracellular routing of rAAV to the nucleus. Additionally, LLnL action is independent of the epithelial surface to which it is administered (i.e., serosal application of LLnL augments mucosal infection and vice versa). This indicates that LLnL need not be endocytosed with AAV particles to enhance transduction. Thus, LLnL may act at a specific time following rAAV endocytosis but during endosomal processing. To provide functional support for this hypothesis, a kinetic analysis of LLnL action at various times after infection from the basolateral surface was performed. In these experiments, LLnL was added to the culture medium either at the time of AAV infection or at various time points after infection. Viral-mediated transgene expression was quantified at 24 hour intervals following infection. Augmentation was achieved regardless of whether LLnL was administrated at 0, 24, 48, and 72 hours after viral infection. However, addition of LLnL at 24 or 48 hours gave the strongest level of augmentation. The ability of LLnL to reduce AAV expression appeared to decline by 72 hour post-infection and was completely lost by 15 days after the initial AAV infection (data not shown). Taken together, it appears that after rAAV enters the cell, it may be targeted to an intracellular compartment that is sensitive to proteasome inhibitor-facilitated liberation. In addition, the loss of an LLnL augmentation effect at 15 days post-infection suggests that enhanced transcription, translation, and/or stability of the transgene products do not likely contribute to the mechanism responsible for this observation.

Combined treatment of LLnL and EGTA prevents degradation of internalized rAAV. To further clarify the molecular mechanism(s) responsible for augmentation of rAAV transduction by LLnL, rAAV genomes in infected cells were analyzed by Southern blotting Hirt DNA. Consistent with studies using S^{35} labeled virus, rAAV binding to either surface of polarized airway epithelia was not affected by LLnL treatment. Southern blotting also demonstrated 2 to 7 fold higher viral binding from the basal surface, which varied among different tissue samples (data not shown). The extent of virus internalization was compared after stripping surface bound virus with trypsin. Confirming previous results, a significant amount of rAAV was endocytosed from the apical surface during the infection period, although viral uptake was more active from basolateral surface. LLnL alone also did not substantially prevent enzymatic degradation of the internalized AAV viral DNA, indicating that enhanced viral trafficking into the nucleus might be more important in the observed augmentation by LLnL. However, treatment with both hypotonic EGTA and LLnL substantially increased the amount of virus internalized from apical surface. Since hypotonic EGTA treatment alone only slightly increased persistence of AAV DNA or AAV-mediated gene expression (Duan et al., 1998; Walters et al., 2000) following apical infection, the predominant mechanism responsible for the combined effects of EGTA and LLnL might be due to reduced degradation of the internalized virus and an increased rate of endocytosis. These synergistic effects of EGTA and LLnL augment rAAV transduction from the apical membrane more than 200-fold. Additionally, the conversion of single stranded viral genomes to linear replication or circular forms has been associated with enhanced AAV transduction by adenoviral early gene products or UV irradiation, respectively (Fisher et al., 1996; Sanlioglu et al., 1999; Duan et al., 1999). Southern blots of Hirt DNA from cultures co-infected with Ad.d1802 and rAAV showed LLnL enhanced AAV transduction was clearly not mediated through the formation of linear replication intermediates (4.7 kb band) as seen in the presence of adenoviral E4orf6 protein produced by Ad.d1802 co-infection.

Ubiquitination of viral capsid proteins following rAAV infection in the airway alters the efficiency of transduction. Proteasome-dependent degradation of

ubiquitinated molecules represents a major pathway for disposal of both endogenous and foreign proteins (Schwartz et al., 1999). Several distinct steps in the regulation of this pathway have been identified, including: activation of ubiquitin by its activating enzyme (E1), transfer of the activated ubiquitin to the ubiquitin carrier protein (E2), and subsequent delivery of the activated ubiquitin to the protein substance by ubiquitin ligase (E3). Ultimately, ubiquitinated proteins are degraded by the 26S proteasome through an ATP-dependent process. To test whether enhancement of rAAV transduction by proteasome inhibitors involves liberation of ubiquitinated virus from an endosomal compartment, the extent of ubiquitin side chains on AAV capsid proteins following infection was examined as well as whether treatment with proteasome inhibitors altered the extent of ubiquitination. AAV capsid proteins were immunoprecipitated using anti-VP 1,2, 3 antibody from rAAV infected human polarized airway cells and confluent human fibroblasts at 6 hours post-viral infection. Subsequent Western analysis with anti-ubiquitin specific antibodies demonstrated a significant increase in the cellular level of ubiquitinated AAV capsid in fibroblasts following proteasome treatment. Ubiquitination significantly increased the molecular weight of capsid proteins (63 kd, 73 kd, and 87 kd) to 220-250 kd and is consistent with the size change following ubiquitination for other molecules (Bregman et al., 1996). Unfortunately, the limited amount of virus retrievable from air-liquid interface cultured human airway cells precluded the ability to detect ubiquitinated capsid in this system (data not shown). Nonetheless, confluent primary fibroblasts also demonstrated augmentation (10-fold) of transgene expression following treatment with proteasome inhibitors. Thus, proteasome inhibitors increase rAAV transduction by decreasing the targeting and/or degradation of ubiquitinated AAV in the proteasome. The net result of such proteasome inhibition would be expected to increase the abundance of ubiquitinated viral capsid.

Because a technical limitation in polarized airway model prevented direct detection of ubiquitinated viral capsid, it was determined whether modulation of other steps in the ubiquitin proteasome pathway could also increase rAAV transduction similarly to that seen with proteasome inhibitors LLnL and Z-LLL.

Several dipeptides, such as H-Leu-Ala-OH and H-His-Ala-OH, are known to inhibit ubiquitin ligase E3 (Obin et al., 1999). Application of these ubiquitin ligase inhibitors indeed enhanced rAAV transduction from the basolateral surface of human airway cells. Taken together, data in both fibroblasts and polarized airway epithelia suggest that AAV capsid is ubiquitinated following endocytosis, and that this process is a barrier to rAAV transduction. The most plausible mechanism responsible for the augmentation of rAAV transduction by tripeptide proteasome inhibitors involves the prevention of ubiquitinated virus degradation and/or targeting to the proteasome.

Long-term enhancement of rAAV transduction by proteasome inhibitor *in vivo*. To evaluate the potential utility of proteasome inhibitors for *in vivo* gene therapy, both the toxicity and efficacy of these agents for *in vivo* rAAV mediated gene transfer in the mouse lung was tested. To assess the toxicity of these proteasome inhibitors in mice, 10, 100, and 1000 fold higher effective doses of LLnL or Z-LLL were administered than used to induce gene transfer in polarized airway cells, using both intra-tracheal and systemic (IV) delivery. No toxicity was indicated by histologic evaluation of the lung and liver or was evidenced by the death of animals. To investigate whether these proteasome inhibitors could improve rAAV transduction *in vivo*, AV.LacZ (5×10^{10} particles) was delivered either alone or in the presence of 400 μM Z-LLL by intranasal administration. Mouse lungs were harvested at 3, 10 and 150 days post-infection to evaluate short and long term effects. Proteasome inhibitor treatment from basal surface, or in conjunction with EGTA from apical surface, resulted in pronounced, immediate enhancement on rAAV transduction, however, X-gal staining of the lung tissues at 3 and 10 days post infection demonstrated no detectable transgene expression in either proteasome inhibitor treated or untreated groups. In contrast, significant transduction was achieved at 150 days in Z-LLL treated samples. Targeted transgene expression was predominantly confined to the conducting airways, rather than in the parenchyma. Alveolar cells were rarely transduced. Although on average only about 5.88% of airway cells were transduced by AV.LacZ, and LacZ positive cells were observed throughout the entire conducting airway, a characteristic transduction profile was

evident. The transduction efficiency in larger bronchioles (> 350 mm) reached a mean of 10.36 ± 1.63% of the airway epithelium, while 1.37 ± 0.41% of airways cells in the smaller bronchioles (< 150 mm) expressed the β-galactosidase transgene. The range of transgene expression in distal and proximal airways was 0 to 4% and 5 to 18%, respectively. This transduction profile demonstrating a higher and more consistent transduction in larger airways likely reflects a more uneven delivery of virus to regions of the lung encompassing the smaller airways. Examination of cryo-sections from lungs infected by AV.LacZ alone revealed only 2 lacZ positive cells in a total of 315 airway sections (n=3 animals).

Discussion

Inefficient gene transfer from the apical surface of the airway has been a major obstacle in numerous gene therapy approaches for cystic fibrosis utilizing recombinant adenovirus (Walters et al., 1999; Pickles et al., 1998), adeno-associated virus (Duan et al., 1998), retrovirus (Wang et al., 1998), and non-viral liposome vectors (Chu et al., 1999). It has been generally thought that inefficient viral mediated gene delivery through the apical membrane of airway epithelia is predominantly due to the lack of receptors or co-receptors on this surface.

Molecular analysis of rAAV infection in polarized airway epithelia has revealed several unique findings. First, there is conclusive evidence that the previously reported lack of known AAV-2 receptor and co-receptors (Duan et al., 1999) at the apical membrane of airway epithelia does not abrogate AAV infection. Although transduction (as determined by transgene expression) from the basolateral surface is 200-fold more efficient than from the apical membrane, quantitative and semi-quantitative analyses of viral endocytosis with either S^{35}-labeled virus or Southern blotting have demonstrated that viral uptake from the apical surface is only 2-7 fold less efficient than from the basolateral membrane. Therefore, it is reasonable to assume that previously unidentified alternative receptor/co-receptors and/or receptor-independent mechanism(s) might be involved in AAV uptake from the mucosal surface of the airway.

Polarity is widely recognized to significantly influence endosomal processing of many proteins, and distinct sorting mechanisms have been described

for the apical and basolateral compartments (Odorizzi et al., 1996; Rodriguez-Boulan et al., 1993). The lack of a direct correlation between the efficiency of viral uptake and transgene expression following basolateral and apical infection suggest that additional post-endocytic barriers exist for rAAV mediated gene transfer. Differences in the extent of AAV nuclear trafficking following basolateral versus apical routes of infection suggest that basal and apical cellular compartments possess distinct biologic properties that may influence the polarity of AAV transduction. Endosomal processing barriers to rAAV transduction may not be limited to polarized epithelial cells. In support of this notion, impaired intracellular trafficking of viral particles to the nucleus has been observed in NIH 3T3 cells. In addition, rAAV can remain in an inactive state for as long as 7 days in confluent primary fibroblast cells until rescued by UV irradiation to a functionally active state. Thus, post-endocytic barriers to infection exist in multiple cell types.

In the airway, the major rate-limiting steps in rAAV transduction from the mucosal surface appear to involve inefficient endosomal processing of the internalized virus. Regulated intracellular proteolysis through proteasomes plays a critical role in many physiological and pathological conditions (Schwartz et al., 1999; Kato, 1999). Recent identifications of many specific proteasome inhibitors has set the foundation for pharmacologic intervention in this cellular enzymatic system as a novel therapeutic approach. For example, several cell permeable synthetic tripeptide aldehydes (such as LLnL and Z-LLL used in this study) have been demonstrated to be promising cancer therapy agents or anti-inflammatory drugs (Goldberg et al., 1995; Kloetzel, 1998; Wojcik, 1999). Additionally, the proteasome has been suggested to have antiviral functions in HIV infection (Schwartz et al., 1998), implying that the inhibition of proteasome function could be beneficial in promoting transduction with recombinant viruses. Based on the molecular evidence that apical infection of rAAV in the airway is significantly limited by post-entry events, ubiquitin/proteasome pathways appear to be instrumental in this blockage. The proteasome is commonly know as a compartment for clearance of endogenous and foreign proteins. However, recent studies also suggested that the proteasome system is involved in regulating

endocytosis (Bonifacino et al., 1998; Strous et al., 1999). From the standpoint of gene delivery, proteasome inhibitors have been shown to protect transfected plasmid DNA from degradation (Coonrod et al., 1997). The results described herein clearly demonstrate that rAAV mediated gene transfer to the airway epithelia is also significantly enhanced by proteasome inhibitors. Furthermore, this enhancement is correlated with proteasome inhibitor stimulated viral trafficking to the nucleus. Although proteasome inhibitors increased long-term levels of AAV transduction form the apical surface, their effect on basolateral infection appeared predominantly to alter the rate, rather than the long-term levels, of transduction. These differences highlight fundamentally distinct pathways involved in rAAV transduction from apical and basolateral surfaces.

Several findings also support the notion that ubiquitination of virus following endocytosis may be a critical mechanism for sorting incoming AAV. First, treatment of airway epithelia with proteasome inhibitors know to block ubiquitin-dependent degradation of proteins enhances rAAV gene transfer. Second, inhibition of ubiquitin E3 ligase activity in airway epithelia also enhances transduction. Lastly, rAAV capsid proteins are ubiquitinated following infection in confluent human fibroblasts, and that the extent of this ubiquitination is increased by inhibition of ubiquitin-proteasome degradative pathways.

From an applied standpoint, one of the most important findings in this study is the persistent high level of rAAV transduction induced by proteasome inhibitor in mouse lung. Co-administration of Z-LLL with rAAV increased transgene expression from undetectable levels to 10.36+/-1.63% of proximal bronchial epithelial cells at 150 days post-infection. This level of gene expression is thought to be sufficient for therapeutic correction of CFTR deficiency (Crystal, 1999). The feasibility of this strategy for clinical application is further supported by the lack of a detectable local or systemic toxicity following proteasome inhibitor administration to mice. Furthermore, preliminary studies in several other organs, e.g., heart skeletal muscle and liver, have suggested that ubiquitination of rAAV2 may occur in an organ-specific fashion. The application of proteasome inhibitors in skeletal and cardiac muscle had no effect on either short-term or long-term rAAV mediated

gene transfer. However, application of Z-LLL in the liver (see Example 7) led to a 7-fold increase in rAAV transduction at 1 month post-infection. These findings suggest that tripeptide proteasome inhibitors could be used to increase gene transfer in organs other than the lung, depending on the cell biology of virus processing.

In conclusion, a significant barrier to apical infection in the airway with rAAV-2 lies at the level of endosomal processing and ubiquitination. Modulation of the ubiquitin-proteasome system has revealed innovative strategies to enhance rAAV transduction from the mucosal surface of the airway for gene therapy of cystic fibrosis.

Example 3

Expression of the *Lac*Z Gene in Lung Airway Epithelium and Liver *In vivo*

The *in vivo* activity of rAAV in the presence or absence of an agent of the invention in the lung or liver may be tested using the *Lac*Z gene. A rAAV vector containing the *Lac*Z gene, recombinant AV.LacZ (5×10^{10} particles), was administered to mouse lung either as virus alone in PBS or virus in combination with 40 μM LLnL in PBS. Virus was directly instilled into C57Balb/c mice trachea with a 30 G needle in a total volume of 30 μl. To insure the spread of the virus in mouse lung, 50 μl air was pumped into lung through the same syringe immediately after virus was administrated. Ninety days after infection, lungs were harvested intact and fixed in 4% paraformaldehyde followed by cryosection. AAV-mediated transgene expression was evaluated by 10 μm tissue sections staining forLac Z.

Recombinant AV.LacZ (5×10^{10} particles) was also administered to mouse liver either as virus alone in PBS, virus in combination with 40 μM Z-LLL in PBS, or virus in combination with 20 μM LLnL in PBS. Virus was directly instilled into portal vein of the C57B6 mice. AAV-mediated LacZ transgene expression was evaluated by histology staining at 2 and 4 weeks post infection in frozen tissue sections.

Example 4

Methods to Determine Additional Agents Useful to Enhance rAAV Transduction

A. To screen for agents that enhance rAAV transduction, any number of cells can be used. A range of concentrations of the agent to be tested can be determined based on, for instance desirable profiles of the agent, desirable toxicity profiles of the agent and/or concentration of the agents employed *in vivo*. The usefulness of the cell type chosen for the screen can be confirmed by testing compounds, e.g., proteasome inhibitors such as LLnL and ZLL which are known to increase rAAV transduction. For example, a AAV2 FLAG-Luc vector was employed to transduce HeLa, ferret fibroblasts, IB3 and Huh (liver) cells in the presence or absence of the proteasome inhibitor MG132. MG132 was confirmed to enhance AAV transduction in all cell types tested: HeLa cell transduction was enhanced about 500-fold at 80 μM, and 200-fold at 40 μM, MG132; ferret fibroblast cell transduction was enhanced about 200-fold at 20 μM, and 17-fold at 4 μM, MG132; IB3-1 cell transduction was enhanced about 30 to 70-fold at 20 to 80 μM MG132; and Huh-7 cell transduction was enhanced about 15- fold at 20 to 80 μM MG132. There was no difference in rAAV transduction efficiency in HeLa cells when either DMSO or ETOH was used as a vehicle for MG132.

B. HeLa cells were selected to screen for additional agents that enhance rAAV transduction, although any cell strain or line; or primary cells, may be employed. Agents were selected from various classes, such as anti-inflammatories (e.g., dexamethasone and cyclosporin A), NSAIDs (e.g., ibuprofen), β-adrenergics (e.g., albuterol), antibiotics (e.g., ciprofloxacin, colison, gentamycin, tobramycin, and epoxomycin), lipid lowering agents (e.g., lovastatin, simvastatin and eicosapentaenoic acid), food additives (e.g., tannic acid), viral protease inhibitors (e.g., Norvir, Kaletra, and Viracept), chemotherapeutics (e.g., aclacinomycin A, doxorubicin, doxil, camptothecin, taxol and cisplatin) and protease inhibitors (e.g., chymostatin, bestatin and chloroquine). The range of concentrations of the agents to be tested were selected based on solubility profiles, toxicity profiles and/or concentrations previously employed *in vivo*.

HeLa cells were infected for 2 hours with an MOI of 100 rAAV in the presence of agents, e.g., ritonavir (Norvir) (1, 10 and 100 μM), cyclosporin A (2.5,

25 and 250 μg/ml), epoxomicin (1, 10 and 50 μM), alcacinomycin A (5, 50 or 500 μM), chymostatin (1, 10 and 100 μM), bestatin (1, 10 and 100 μM), doxorubicin (adriamycin) (0.1, 1 and 10 μM), camptothecin (camptosar) (1, 10 and 100 μM), eicosapentanoic acid (1, 10 and 100 μM), tannic acid (2, 20, 200 and 2000 μM), simvastatin, prodrug (2, 20 and 200 μM), cisplatin (0.2, 2 and 20 μg/mL), and chloroquine (4, 40 and 400 μM). Forty-eight hours after infection, cells were harvested for analysis. rAAV transduction was measured by removing the media from the cell cultures, adding 100 μL reporter lysis buffer (RLB) and freezing. The supernatant was thawed and transferred to microfuge tubes, freeze thawed an additional 2 times, clarified by centrifugation for 10 minutes and then analyzed for reporter gene expression on the lumometer. Protein was determined by Bradford analysis and results were expressed as relative light units per mg protein (RLU/mg). Data is presented in Figures 1A-E.

Doxorubicin, epoxomicin, and camptothecin all showed a dose-dependent increase in transduction at the dose ranges tested. At the doses tested doxorubicin and epoxomicin increased transduction efficiency up to 169-fold and 120-fold, respectively, camptothecin increased transduction efficiency by 15-fold, tannic acid increased transduction efficiency by 17-fold, cisplatin increased transduction efficiency by 16-fold, and simvastatin increased transduction efficiency by 4-fold.

It should be noted with respect to simvstatin and the lovastatin, that these drugs are formulated as prodrugs and conversion to the activated open ring forms was not confirmed which may have contribute to the negative results. Similarly, the liposomal formulation of doxorubicin, doxil could not be confirmed to be bioavailable to cell culture cells. Thus, agents which initially screened as statistically negative may be reflective of formulations that are not readily bioavailable to cell culture cells or may be reflective of the limited dose range or exposure time.

Epoxomicin, a naturally occurring antibiotic isolated from Actinomycetes known to inhibit NF-KB-mediated signaling *in vivo* and *in vitro*, inhibits proteasomes by inhibiting a proteasome-specific chymotrypsin-like protease. Doxorubicin, an anti-tumor antibiotic which inhibits topoisomerase II and inhibits

nucleic acid synthesis, is translocated by a 20S proteasome from the cytoplasm to the nucleus. Camptothecin, a reversible DNA topoisomerase inhibitor, down regulates topoisomerase via an ubiquitin/26S proteasome pathway. Simvastatin is an agent that modulates proteasome activity, tannic acid inhibits chymotrypsin-like activity and is a cancer chemopreventative, and cisplatin is a chemotherapeutic which crosslinks DNA.

C. To determine whether combinations of agents that enhance rAAV transduction efficiency have synergistic or additive effects when used in combination, cells were contacted with the proteasome modulator, doxorubicin, and the proteasome inhibitor Z-LLL or LLnL. Different AAV vectors were tested, including splicing vectors and pseudotyped rAAV. Viral stocks utilized were as follows: Av2RSVluc, 5 x 10^8 particle/μl; Av2RSVlucCap5 (also referred to as Av2/5 CMVLuc), 2 x 10^9 particle/μl; Av2CMVluc, 1.3 x 10^9 particle/μl; and Av2CMVlucCap5, 1.1 x 10^9 particle/μl. Combinations of agents were compared to the agents used alone to determine the efficiency of transduction. LLnL was used at 40, 200 or 400 μM, Z-LLL at 4 μM and doxorubicin at 0.5 or 1 μM when employed alone. When a combination of LLnL and doxorubicin was used, LLnL was used at 4, 10, 20, 40, 200 or 400 μM and doxorubicin at 1 or 5 μM. The apical surface of polarized airway epithelia, HeLa cells or ferret fibroblast was contacted with the agents and rAAV (5 x 10^9 particles per well).

The results showed that LLnL enhances transduction in HeLa, ferret fibroblast and polarized epithelial cells at 40 μM and A549 cells at 200 to 400 μM. Doxorubicin enhanced transduction in HeLa and ferret fibroblast cells at 1 μM and A549 or polarized airway cells at 5 μM, and enhanced transduction about 100 fold when ferret fibroblasts were infected with lacZ splicing vectors. Doxorubicin also enhanced AAV2 and AAV5 transduction to a greater extent than LLnL. Synergistic effects were noted when doxorubicin and LLnL were co-administered.

In the absence of agent administration, transduction from the apical surface of polarized epithelial cells was greater with AAV vectors with AAV5 capsid than AAV vectors with AAV2 capsid. In the presence of doxorubicin, a 200 to 600-fold induction was observed for AAV2 and AAV5 apical infection of polarized cells.

Thus, agents of the invention can enhance rAAV transduction, including in serotype, pseudotype and multiple vector strategies.

D. Endotracheal administration of 10^{11} AV2FLAG-luc rAAV particles to male Balb/c mice in conjunction with intravenous administration of Doxil (dosed in a range of 2, 10, or 20 mg/kg), a liposomal preparation of doxorubicin, to mice enhanced AV2FLAG-luc transduction by 2 logs by day 7 at the 20 mk/kg dose of doxil. Specifically, at 20 mg/kg doxil, transduction was enhanced on the average of 67-fold by day 7 and 4-fold by day 30. It is worth noting that doxil previously tested negative in cell line screening while the free compound doxorubicin tested positive in cell line screening (Figures 1A-E). Liposomal formulations have desirable properties for *in vivo* use including their increased stability or circulation half life making them more bioavailable *in vivo*. Those same characteristics make liposomal formulations less desirable for *in vitro* screening as described above. Thus, one skilled in the art can design formulation strategies for agents of the invention to tailor them to the desired application. In addition to formulation design, one skilled in the art can tailor routes of delivery in order to maximize rAAV transduction efficiencies.

In additional experiments, a pseudotyped rAAV vector encoding FVIII was tested in male Rag-1 mice. Rag-1 mice were used because as described in the art, normal mice produce inhibitors of human FVIII that can obscure protein detection in the serum. Rag-1 mice are known to be deficient in the pathways necessary to produce these inhibitors and thus will either produce no inhibitors, lower levels of inhibitors or have extended time periods for development of inhibitors. The rAAV vector was constructed containing serotype 5 capsid proteins and 5'-3' ITRs of AAV-2 flanking a heterologous transgene comprised of the minimal liver specific element HNF3/EBP and a human B-domain deleted FVIII gene (a second construct was identical except it contained a B-domain deleted canine FVIII gene). Animals were administered 10^{12} rAAV vector particles intravenously via the lateral teil vein concurrently with 20 mg/kg of doxil at day 0. Circulating, bioavailable FVIII activity was measured from the serum at days 31, 53 and 90 by techniques known in the art including ELISA and Coatest. Data presented in Figure 3 demonstrate that

animals not treated with doxil had barely detectable levels of FVIII in the range of 0.99 ng/ml for days 31 and 53 which decreased to 0.13 ng/ml by day 90. In contrast, animals dosed with 20 mg/kg of doxil had over 40 times the levels of FVIII protein. Interestingly, the decline in FVIII protein seen in animals not treated with doxil at day 90 (0.13 ng/ml) was not evident in animals treated with doxil (40.16 ng/ml) indicating that doxil not only enhanced rAAV transduction as evident at the shorter time period, but the agent of the invention also prolonged expression. In order to demonstrate that doxil was affecting rAAV transduction and not merely affecting the FVIII protein translation or stability, RS-PCR was performed on liver tissue at the day 53 time point. The data presented for individual animals in Table 1 demonstrates that the increase in FVIII protein noted in animals treated with doxil correlates with the levels of mRNA detected.

The increase *in vivo* rAAV transduction produced by doxil was further confirmed utilizing the same vectors and protocol described above in male FVIII knockout mice tolerized to the human FVIII protein utilizing a cytoxan mediated tolerization strategy as described in the art. Animals were treated with weekly injection of 50 mg/kg cytoxan beginning at the time of rAAV vector delivery. Data presented in Table 2 confirmed the previously described results when tested by ELISA or Coatest at days 14 and 25, namely animals dosed with doxil demonstrated at least a ten-fold enhancement of rAAV transduction.

Table 1

Animal Number	Treatment	Molecules FVIII mRNA/cell	FVIII Protein (ng/mL)
#26	AAV2/5 HNF3/EBP	2.15	0.68
#27		0.91	<0.63
#28		1.98	0.97
#29		2.06	1.45
#30		2.45	0.77
#31		2.29	<0.63
#59	AAV2/5 HNF3/EBP FVIII + Doxil	65.47	31.85
#60		41.4	37.75
#61		99.43	51.9
#62		49.44	38.65
#63		43.9	40.55
#64		57.54	31.55
Animal Number		Molecules FVIII mRNA/cell	FVIII Protein (ng/mL)
#26		2.15	0.68
#27		0.91	<0.63
#28		1.98	0.97
#29		2.06	1.45
#30		2.45	0.77
#31		2.29	<0.63
#59		65.47	31.85
#60		41.4	37.75
#61		99.43	51.9
#62		49.44	38.65
#63		43.9	40.55
#64		57.54	31.55

Table 2. *In Vivo* Enhancement of FVIII RAAV Transduction

Day 14 Results

Sample		Final Result (DF*ng/mL)	Coatest (mU/mL)
Group 1 Vehicle	Animal #		
	801	< 0.63	0
	804	< 0.63	0
	805	< 0.63	0
	847	< 0.63	0

Group 2 AAV2/5-HFN3/EBP-FVIII		
	816 < 0.63	0
	817 < 0.63	0
	818 0.92	0
	819 < 0.63	0
	820 < .63	0
	834 0.9	0
Group 2 AAV2/5-HFN3/EBP-FVIII + Doxil		
	870 60.45	171
	871 26.29	0
	872 12.395	14
	873 44.3	30
	874 12.135	122
	875 31.04	94

2.X.10, Day 25 FVIII ELISA

Sample	Final Result	
Group 1 Vehicle		
	806 < 0.63	0
	807 < 0.63	0
	808 < 0.63	0
	849 < 0.63	0
Group 2 AAV2/5-HFN3/EBP-FVIII		
	821 < 0.63	0
	822 < 0.63	0
	823 < 0.63	0
	824 1.27	0
	825 0.72	0
	833 0.74	0
Group 3 AAV2/5-HFN3/EBP-FVIII + Doxil (no spikes)		
	841 16.785	49.833
	842 12.425	37.282
	843 13.685	41.466
	844 35.225	91.842
	845 7.815	12.974
	846 24.02	54.853

Thus, agents that interact with molecules in intracellular AAV trafficking pathways, such as proteasomes or molecules in the ubiquitin pathway, by binding to

those molecules and/or inhibiting their activity, are useful to enhance rAAV transduction.

Example 5

Proteasome Involvement in rAAV-2 and rAAV-5 Transduction of Polarized Airway Epithelia *In vitro* and *In vivo*

Inhibition of the proteasome with small tripeptide inhibitors such as LLnL can significantly augment rAAV-2 transduction from the apical membrane of both polarized human airway epithelia *in vitro* and mouse lung *in vivo* (Duan et al., 2000). As AAV-5 has been reported to have higher tropism for, and alternate receptors on, the apical membrane of airway epithelia, increased transduction of airway epithelia from the apical membrane with rAAV-5 might be due to altered proteasome involvement. Co-administration of a proteasome modulator and a proteasome inhibitor was found to augment transduction of both serotypes in a cell type dependent manner.

To better understand serotype-specific differences in airway transduction, the effect of proteasome inhibitors on rAAV-2 and rAAV-5 transduction in polarized human airway epithelial cultures and mouse lung was examined (Figures 2 and 6). A proviral construct containing 5' and 3' ITRs from AAV-2 flanking a transgene was packaged into both AAV-2 and AAV-5 capsid to generate AV2.RSVluc and AV2.RSVlucCap5 viruses which express the luciferase transgene. rAAV-2, but not rAVV-5, demonstrated a significant difference in transduction from the apical versus basolateral surface. Transduction with AV2.RSVluc was 36- and 103-fold greater from the basolateral membrane at 5 and 14 days post-infection, respectively. In contrast, AV2.RSVlucCap5 transduced epithelia from the apical and basolateral membranes with similar efficiencies at both time points.

LLnL augments AV2.RSVluc transduction from the apical and basolateral surfaces. However, application of LLnL selectively increased AV2.RSVlucCap5 transduction 12-fold only when virus was applied to the apical surface. These results suggest an interesting difference in the involvement of the proteasome for various AAV capsid entry pathways that are effected by cell polarity.

The proteasome inhibitor Z-LLL was found to induce long-term (5 month) transduction with rAAV-2 in mouse lung. To determine *in vivo* transduction efficiency of AV2.RSVlucCap5, mice were infected with 6 x 10^{10} particles of AV2.RSVlucCap5 by nasal aspiration alone (control) or in combination with 200 μM Z-LLL, 200 μM doxorubicin or 200 μM Z-LLL and 200 μM doxorubicin (12 mice per group). Co-administration of Z-LLL induced whole lung luciferase expression 17.2- and 2.1-fold at 14 (2 weeks) and 42 (6 weeks) days post-infection, respectively. Interestingly, luciferase expression was further reduced at 3 months post-infection (Figure 2).

Co-administration of doxorubicin induced whole lung luciferase expression at levels almost ten times higher than those for Z-LLL at 2 weeks. Doxorubicin also induced tracheal and bronchi luciferase expression at higher levels than Z-LLL at 2 weeks. At six weeks, a similar pattern was observed for Z-LLL and doxorubicin alone, however, luciferase levels were more than additive in trachea and bronchi in mice co-administered virus, Z-LLL and doxorubicin. By three months post-infection, the synergism was no longer observed. These observations suggest a striking difference in the kinetics and longevity of induction by Z-LLL between *in vivo* studies with rAAV-2 and rAAV-5. Since *in vivo* transduction is significantly more efficient with rAAV-5 compared to rAAV-2, altering proteasome activity may simply enhance the rate of transduction with rAAV-5. In the case of rAAV-2, this basal rate may be significantly reduced from the apical membrane *in vivo* rendering more sustained augmentation of transduction by proteasome inhibitors.

These results also highlight the use of different agents and vectors to achieve different results. For example, agents and vectors that result in a steady increase in transgene expression in particular cells over time may be useful for certain disorders or conditions while agents and vectors that result in a high burst of transgene expression may be useful for metabolic disorders such as hemophilia.

Ubiquitination and proteasome activity can influence a myriad of intracellular processes that control protein degradation and intracellular trafficking. The following examples are designed to identify the molecular mechanisms of rAAV transduction that are controlled by the ubiquitin/proteasome system. These

studies may lead to a clearer understanding of pathways and/or molecules that influence rate-limiting steps in rAAV transduction and can also be used to identify further useful agents to enhance processing of rAAV (i.e., endosomal escape, trafficking to the nucleus, and uncoating) and hence transduction.

Example 6

Dual Fluorochrome Labeling of rAAV to Follow Endosomal Escape

One of the most challenging but important aspects of intracellular trafficking of rAAV is determining the exact endosomal compartment from which virions exit into the cytoplasm. Proteasome inhibitors may modulate this aspect of the rAAV life cycle by either changing the rate of endosomal escape and/or the compartment from which rAAV enters into the cytoplasm.

Methods

To study endosomal escape, single-cell imaging and microinjection of quenching antibodies against one of two fluorochromes on a dual-labeled rAAV capsid were performed. The Alexa Fluor system from Molecular Probes was chosen as a system for which multiple fluorochromes could be linked to the rAAV capsid at similar efficiencies. Three dyes (Alexa Fluor® 488 [green], Alexa Fluor® 568 [Red] and Alexa Fluor® 647 [blue]) were selected as useful in this regard. Preferably, dual labeling of rAAV does not change the infection pattern. Also preferably, microinjection of quenching antibodies against Alexa-488 (Molecular Probes) can shift fluorescence of dual-labeled rAAV. The general approach to assess endosomal escape is to inject the cytoplasm of living cells with anti-Alexa-488 following infection with rAAV that is dual labeled with Alexa-488 and one of the other dyes. Alexa-488/568 dual-labeled rAAV, a shift in fluorescence of virus from yellow to red (i.e., quenching of the green fluorochrome) indicates movement of virus into the cytoplasm. This approach is used in combination with GFP-tagged endosomal compartments and/or dominant negative Rabs to evaluate the compartment from which rAAV moves into the cytoplasm.

Alexa labeling of rAAV. The monovalent Alexa succinimidyl ester reactive dye (Alexa-488 and/or Alexa-568) was dissolved in 50 µl of 1 M bicarbonate. 0.5 $x10^{12}$ particles (determined by slot blot) of purified AV2Luc in 0.5 ml Hepes buffer

was added to the reaction mixture and incubated for 2 hours. When dual labeling was performed, equal molar amounts of the two fluorochromes was used and the reaction time was extended to 3 hours. The labeled rAAV2 was separated from the free dye by exclusion chromatography. The fractions were tested for infectious titers on HeLa cells using luciferase assays. The 5 peak fractions were then combined and used for fluorescent imaging studies. Imaging studies were performed.

Results and Conclusions

Assessment of functional particles demonstrated that greater than 85% activity was retained following label with Alexa dyes (data not shown). This was similar to results observed with Cy3 labeling. Results from Hela cells infected with Alex-568-labeled rAAV2 demonstrated a significant overlap in signal with the GFP-tagged Rab11 compartment. The distribution observed was very similar to that seen with Cy3-labeled rAAV2. From these studies, it was concluded that Alexa-labeling of rAAV can be performed, and it was slightly more sensitive than Cy3-labeling. In these studies, approximately 3-4 fluorochromes were labeled on each rAAV capsid. To investigate whether dual labeling procedures could also be adapted to efficiently label rAAV, studies were conducted that compared dual Alexa-488/568 and Alexa-568-labeled rAAV2 following a 1 hour infection of Hela cells. These studies, which demonstrate overlap in the Alexa-488/568 signal, as compared to Alexa-568 alone, confirm that the predominance of rAAV virions are dual-labeled when both dyes are added to the conjugation reaction.

To begin to develop assays for visualizing endosomal release of rAAV into the cytoplasm, it was determined that single cell injection of Anti-Alexa-488 into Hela cells infected with AV2Luc could quench green fluorescence from dual-labeled Alexa-488/568 once rAAV entered into the cytoplasm. Moreover, the level of Alexa-488 fluorescence was significantly quenched by injection of anti-Alexa-488 while leaving red channel fluorescence of Alexa-568 intact. In contrast, fluorescence of both fluorochromes remained quite high in uninjected cells. The remaining Alexa-488 fluorescence in injected cells was interpreted as virus still remaining in the endosomal compartment protected from antibody binding. These

findings suggest that a significant portion of rAAV may be free in the cytoplasm by two hrs post-infection.

Example 7

Altered Trafficking of rAAV

Proteasome-modulating agents act to increase rAAV transduction through one or more of the following mechanisms: 1) increasing the rate at which rAAV accumulates in the primary compartment through which it emerges to the cytoplasm without changing the pathway of intracellular trafficking; 2) altering the pathway of rAAV intracellular trafficking in a manner that leads to more efficient accumulation in a compartment through which it emerges to the cytoplasm; 3) increasing the efficiency at which rAAV breaks out of the endosomal compartment; and/or 4) enhancing the rate of nuclear trafficking of free rAAV in the cytoplasm.

Several lines of evidence suggest that proteasome inhibitors may act to enhance rAAV transduction by increasing the rate of viral transport to the nucleus (Duan et al., 2000) and/or enhancing viral processing of the capsid (Yan et al., 2002). First, proteasome inhibitors such as the tripeptides LLnL and Z-LLL enhance transduction of both rAAV2 or rAAV5, viruses without enhancing 1) endocytosis of virus, 2) stability of viral DNA within the cell, or 3) promoter activity which drives transgene expression (Duan et al., 2000; Yan et al., 2002). Second, proteasome inhibitors can be added up to a week following infection of polarized human airway epithelia and still enhance transduction (i.e., gene expression). Third, viral capsids for type 2 and type 5 show enhanced ubiquitination *in vivo* in the presence of proteasome inhibitors, and purified virus can also be ubiquitinated *in vitro* (Yan et al., 2002). Together, these findings strongly suggest that modulating proteasome activity enhances rAAV transduction for at least two serotypes and that the mechanism of enhancement involves some aspect of intracellular viral processing.

A. Proteasome Inhibitors Increase Transport of rAAV2 and rAAV2/5 cell to the Nucleus

A large number of various classes of proteasome inhibitors were screened to identify those that had the largest effect. Two classes of compounds (the tripeptidyl aldehyde LLnL and an anthracyclin derivative doxorubicin), and their ability to induce rAAV2 and rAAV2/5 transduction in two airway cell lines (IB3 and A549) are described below.

Methods

LLnL and Z-LLL are two tripeptidyl aldehydes shown to inhibit calpains, cathepsins, cysteine proteases as well as the chymotrypsin-like protease activity of proteasomes (Wagner et al., 2002; Donkor, 2000; Sasaki et al., 1990). Doxorubicin has also been shown to inhibit chymotrypsin-like protease activity of proteasomes (Kiyomiya et al., 2002). Both classes of proteasome inhibitors bind tightly to the proteasome complex. Dose response curves for these two proteasome-modulating agents were evaluated on IB3, A549, Hela, and primary fibroblasts. The responses were consistent for a number of cell lines and for three different promoters driving luciferase expression. For one set, CMV-driven luciferase constructs with an AAV2-based genome were employed that were packaged into AV2 or AV5 capsids. Cells were infected at various doses of AV2Luc and AV2/5Luc (MOIs 100 to 1000 particles/cell). At the time of infection, cells were treated with various concentrations of LLnL or Doxorubicin and gene expression was assayed at 24 hours post-infection. The effect of proteasome inhibitors on nuclear uptake of virus was evaluated using a previously-described protocol for fractionating viral DNA in the cytoplasm and nucleus (Xiao et al., 2002). Viral DNA content in the cytoplasmic and nuclear fractions was then evaluated by slot blot hybridization against a Luciferase DNA probe.

Results and Conclusions

Results from this analysis demonstrated that both LLnL and Dox can significantly augment rAAV2 and rAAV2/5 transduction in two independent airway cell lines (Figure 3). Although the trends were similar between these two cell lines and the two serotypes of rAAV, several features of the induction are worth noting. First, transduction in IB3 cells was most significantly inducible (> 200-fold) by

LLnL, while A549 cells required much higher concentration of LLnL to achieve 10-fold lower levels of induction. Hence, IB3 cells appear to be particularly sensitive to LLnL induction of rAAV. Second, rAAV transduction in both cell lines was highly inducible (200-fold) by Dox.

Given previous findings in polarized human airway epithelial cells that treatment with LLnL increased movement of rAAV to the nucleus (Duan et al., 2000), it was determined whether LLnL and Dox treatment at the time of infection also enhanced rAAV movement to the nucleus. Subcellular fractionation of nuclei and cytoplasmic extracts from rAAV2-infected IB3 cells, demonstrated that both Dox and LLnL significantly increased the fraction of viral DNA in the nuclear compartment. These findings suggest that these two proteasome-modulating agents act to increase rAAV transduction by mobilizing virus to the nucleus. In summary, these findings support a growing body of work that the ubiquitin/proteasome system acts in some manner to control intracellular processing of rAAV and its movement to the nucleus.

B. LLnL and Dox Act through Distinct Mechanisms to Modulate the Proteasome and Enhance rAAV Transduction.

To test the hypothesis that LLnL and Dox might augment rAAV transduction through distinct mechanistic interactions with the proteasome, their effects on rAAV transduction were assessed when added in combination. If each of these drugs acted to augment transduction by distinct mechanistic interactions with the proteasome, then their cumulative effect would be greater than either individually.

Methods

Hela, A549, IB3, and primary fetal fibroblasts were evaluated for AV2Luc and AV2/5Luc transduction in the presence of LLnL, Dox, or LLnL + Dox at various concentrations. The data shown is from Hela and A549 cells at the most optimal dose combination that induces rAV2Luc transduction to a greater extent than each compound alone.

Results and Conclusions

Findings in Figure 4 demonstrated that cooperative inhibition of the proteasome by multiple proteasome inhibitors can provide increased augmentation

in rAAV transduction. The observation that combined Dox and LLnL treatment enhances rAAV transduction greater than either compound alone does not, in and of itself, prove that the mechanisms of induction are independent of one another. There are several potential reasons why such drugs might cooperatively enhance rAAV transduction. First LLnL and/or Dox might alter endosomal routing of rAAV, enhance endosomal escape, and/or mobilize rAAV in the cytoplasm to the nuclear pore. Each of these agents might affect any one or more of these processes to differing extents and allow for additive or synergistic affects on rAAV transduction. Hela cells appear to provide a greater additive effects of Dox and LLnL on rAAV transduction than A549 cells. Furthermore, it should be noted that in primary fetal fibroblasts, no additive effect on transduction is seen (data not shown). In this cell line, Dox most significantly enhances transduction of rAAV2 and rAAV5, and LLnL provides no additional induction despite the fact it induced transduction 10-fold by itself. These interesting cell-specific differences also imply that certain cellular processes that alter rAAV transduction may be uniquely controlled by LLnL and Dox interactions with the proteasome.

Example 8

Dual Therapeutic Uses of Proteasome Modulating Agents

Methods

Primary cultures of human CF bronchial epithelia and rAAV infection. Airway epithelial cells isolated from bronchial tissue obtained from CF or non-CF patients were seeded onto collagen-coated, semi-permeable membranes (0.6 cm^2 Millicel-HA; Millipore, Bedford, MA). Methods to generate these air/liquid interface cultures and the medium used were as described in Zabner et al. (1998). Four viral vectors, AV2CF83, AV2tgCF, AV2/5CF83, and AV2/5tgCF, were used to infect polarized airway epithelial cells from the apical membrane. All vectors harbored AAV2 ITRs and were either packaged into type 2 or type 5 capsids using a triple plasmid transfection technique, and purified by ion exchange chromatography as described in Kaludov et al. (2002). AV2tgCF is the current clinically-used AAV2-based full-length CFTR vector in which expression of CFTR is driven off the ITR (Aitken et al., 2001; Wagner et al., 2002). AV2/5tgCF virus has the

identical proviral structure to AV2tgCF, but is pseudotyped into AAV5 capsid. AV2CF83 and AV2/5CF83 viruses have an additional 83 bp minimal promoter (Lynch et al., 1999) inserted into the AV2tgCF proviral genome to increase gene expression, and were packaged into AAV2 and AAV5 capsids, respectively. The airway epithelial cultures were infected with 75 μl of virus-containing medium applied to the apical surface of the epithelia at an MOI of 10^5 particles/cell in the presence or absence of 40 μM LLnL and 5 μM doxorubicin. The cells were incubated at 37°C for 16 hours before apically loaded virus was removed and the epithelia were returned to 0.6 ml of basolateral medium and air at the apical interface in the absence of proteasome modulating agents. Cultures were then incubated for an additional 15 days (changing basal media every two days) prior to electrophysiologic and molecular studies. Transepithelial resistance was monitored prior to viral infection and following the 15 day post-infection incubation to confirm epithelial integrity. A transepithelial resistance >500 Ohms was used to indicate that the electrical integrity of the epithelium had not deteriorated over the course of the experiment. In addition to the above primary CF airway model system, polarized CF airway epithelia were similarly infected using the transformed CuFi cell line as described in Zabner et al. (2003).

Short-circuit current (Isc) measurement in polarized airway epithelia. Transepithelial short-circuit currents were measured using an epithelial voltage clamp and a self-contained Ussing chamber as described in Zabner et al. (1998). The basolateral side of the chamber was filled with Ringer's buffer solution containing 135 mM NaCl, 1.2 mM $CaCl_2$, 2.4 mM KH_2PO_4, 0.2 mM K_2HPO_4, 1.2 mM $MgCl_2$, and 5 mM HEPES, pH 7.4. The apical side of the chamber was filled with a low-chloride Ringer's containing 135 mM sodium gluconate, 1.2 mM $CaCl_2$, 2.4 mM KH_2PO_4, 0.2 mM K_2HPO_4, 1.2 mM $MgCl_2$, and 5 mM HEPES, pH 7.4. During the experiment, the chamber was maintained at 37°C and aerated with 100% O_2. Transepithelial voltage was clamped at zero, and the resulting Isc was measured and recorded following the sequential addition of the following channel antagonist and agonists: 1) 100 μM amiloride (apical), 2) 100 μM 4,4′-diisothiocyna-2,2′-disulfonic stilbene (DIDS) (apical), 3) 100 μM IBMX/10 μM forskolin (apical), 4) 100 μM bumetanide (basolateral). Voltage was referenced to the apical

compartment. The series resistance of the Ringer's solution and transwell membrane was electrically compensated before starting experiments. All chemical agonists and antagonists were added to either the apical or the basolateral sides of the monolayer by direct injection and mixed by aerating the Ringer's solution. After the experiment, membranes were harvested and stored at –80°C.

RNA Processing and RNA-Specific PCR. Transgene-derived recombinant CFTR mRNA and endogenous CFTR mRNA were quantified using an RNA Specific real-time reverse transcriptase PCR (RS-PCR) method recently described and currently used in the Targeted Genetics Inc. clinical trails for CF (Gerard et al., 2003). Total RNA was isolated from cells growing on Millicel-HA membranes using the RNeasy column purification method (Qiagen, Valencia CA). Specifically, 350 μL of RNeasy lysis buffer (RLT + β-mercaptoethanol) was added directly to harvested membranes in a microfuge tube and samples were vortexed for 15 seconds. The lysate was then removed and passed through a Qiashredder (Qiagen, Valencia CA) after which the standard mini-column protocol was followed and RNA was eluted in 30 μL of TE (10 mM Tris pH 8, 1 mM EDTA). Quantitation was by absorbance at 260 nm. All cDNA samples were tested in duplicate by RS-PCR for rAAV transgene derived CFTR, endogenous CFTR, and β-glucuronidase (GUS). Both rAAV and endogenous CFTR expression were normalized to the GUS endogenous control. This method allows direct relative comparison of the level of rAAV CFTR expression to endogenous CFTR expression.

DNA Processing and Real-Time DNA PCR for viral genomic DNA. Cellular DNA was recovered by ethanol precipitation from a pool consisting of the RNeasy column load flow-through fraction and the first column rinse (from RNA processing). This allowed for a direct comparison of vector DNA and RNA for a given sample. The recovered DNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated in 2.5 volumes of ethanol and quantitated by absorbance at 260 nm. Seventeen of the 90 DNA isolations were chosen at random and screened for matrix inhibition by evaluating DNA spike recovery; there was no evidence of matrix inhibition (data not shown). All test samples were analyzed in a real-time quantitative TaqMan PCR assay targeting

AAV-CFTR (vector-specific) sequences. Each 20 μL reaction contained 200 ng of genomic DNA and was run in triplicate in a 384-well format using an ABI Model 7900 Sequence Detection System (Applied Biosystems, Foster City CA). Standards consisted of the plasmid ptgAAVCF (containing the AAV-CFTR sequence) diluted into a background of normal human lung DNA (Clontech/BD Biosciences, Palo Alto CA) and ranged from 8×10^6 to 8×10^1 copies per PCR. AAV CFTR-specific PCR primers and Taqman probe were as follows: forward 5'-TTGCTGCTCTGAA AGAGGAGAC-3' (SEQ ID NO: 1); reverse 5'-GATCGATGCATCTGAGCTCTTTAT-3' (SEQ ID NO:2); probe 5'-(FAM)TGCTGCTCTCTAAAGCCTTGTATCTTGCACC(TAMRA)-3' (SEQ ID NO:3).

Quantitative RT-PCR for different ENaC subunits. Following CFTR mRNA quantification by RS-PCR, total RNA samples were used to generate cDNA (Invitrogen). The following primers and probe sequences were used for TaqMan PCR quantification of ENaC subunits: α–ENaC subunit: forward 5'-CCTCAACTCGGACAAGCTCG-3' (SEQ ID NO:4); reverse 5'-GAGAGTGGTGAAGGAGCTGTATTTG-3' (SEQ ID NO:5); probe 5'-(FAM)ACCCTCAATCCCTACAGGTACCCGGAAATT(TAMRA)-3' (SEQ ID NO:6). β–ENaC subunit: forward 5'-GGAACCACACACCCCTGG-3' (SEQ ID NO:7); reverse 5'-CAAAGAGATCAAGGACCATGGG-3' (SEQ ID NO:8); probe 5'-(FAM)CCTTATTGATGAACGGAACCCCCACC(TAMRA)-3' (SEQ ID NO:9). γ–ENaC subunit: forward 5'-GCTGGATTTTCCTGCAGTCAC-3' (SEQ ID NO:10); reverse 5'CAGGGCCTCTCTGGTCTCCT-3' (SEQ ID NO:11); probe 5'-(FAM)AACATCAACCCCTACAAGTACAGCACCGTTC(TAMRA)-3' (SEQ ID NO:12). Copies of ENaC subunit mRNA were normalized to the number of β-actin mRNA copies in each sample using commercially available primer sets from Applied Biosystems (Foster City, CA).

Analysis of γ-ENaC promoter CpG Methylation. The methylation status of a CpG island beginning at approximately -1.8 kb of the γ-ENaC promoter was analyzed using a previously described PCR method (Malik et al., 2001). Briefly, genomic DNA was isolated from A549 cells that had been treated with and without

doxorubicin, and then digested overnight with MboI, MboI/MspI, or MboI/HpaII. PCR reactions were then performed using primers that flank the MspI/HpaII sites in this region. The relative positions of the CpG island, restriction sites, and primers are shown in Figure 11B. Primers: forward 5'-TTGGAACCGAAAGGTGAGTT-3' (SEQ ID NO:13); reverse 5'-TGAACAGGCGCTGGGCGGAG-3' (SEQ ID NO:14).

Results

Proteasome modulation agents enhance rAAV-mediated CFTR functional correction in polarized CF airway epithelia. Proteasome inhibitors have been shown to dramatically increase the transduction efficiency of rAAV infection from the apical surface of polarized human airway epithelia (Duan et al., 2000; Ding et al., 2003). This enhancement by proteasome inhibitors appears to reflect an increased efficiency of intracellular processing of rAAV and accumulation in the nucleus (Duan et al., 2000) while not affecting processes that directly control the efficiency of second strand synthesis (Ding et al., 2003). Hence, proteasome inhibitor action on rAAV transduction in polarized airway epithelia suggests that increased functional conversion of single-stranded genomes to expressible forms is facilitated by the increased bulk flow of rAAV into the nucleus. Based on these findings, the efficacy of both the current clinically used ITR driven full-length CFTR rAAV vector (AV2tgCF, also called tgAAV2-CF), and a second generation vector harboring a short 83 bp synthetic promoter (see U.S. Patent No. 6,346,415) driving expression of a full-length CFTR cDNA (AV2CF83), were assessed for their ability to correct CFTR chloride transport in human CF airway epithelia in the presence of proteasome modulating agents. Additionally, since rAAV5 has been suggested to transduce the apical surface of human airway epithelia more efficiently than rAAV2, pseudotyped rAAV2/5 viruses with both types of vector genomes were also evaluated.

rAAV2 or rAAV2/5 vectors utilizing the ITR or synthetic promoters were used to infect polarized CF airway epithelia from the apical surface in the presence or absence of a combined cocktail of LLnL and doxorubicin. Fifteen days following infection, CFTR-mediated cyclic AMP (cAMP)-sensitive short-circuit current (Isc) was assessed after stimulation by IBMX (100 μM) and forskolin (10 μM), and

compared to normal human airway epithelia. In total, samples from 3 different CF donors (CFB-16, CFB-19, CFB-26) were infected and analyzed for CFTR correction. Results from these experiments are shown in Figure 7. In the absence of proteasome inhibitors, only minimal restoration of cAMP-inducible chloride currents were seen with the minimal promoter vector (AV2CF83) of the type-2 serotype (0.76 +/-0.16 μA) and no significant functional correction was seen with any of the other three viruses tested (AV2tgCF, AV2/5tgCF, or AV2/5CF83). However, when proteasome inhibitors LLnL and doxorubicin were provided only at the time of infection, AV2CF83 restored CFTR-mediated chloride current upon IBMX/forskolin stimulation in the CF epithelia at the highest level (2.9+/-0.3 μA), reaching approximately 80% of that seen in normal human airway epithelial (3.5+/-0.8 μA). Surprisingly, pseudotyped AV2/5CF83 with the same synthetic promoter gave significantly less correction of chloride currents in CF epithelia (1.0+/- 0.2 μA), and was even lower than that seen with the ITR promoter CFTR vector AV2tgCF (1.9+/-0.2 μA). For each serotype, the addition of the 83 bp synthetic promoter significantly increased ($p < 0.03$) IBMX/forskolin responsive Isc as compared to ITR-driven CFTR vectors.

Functional activity of intracellular viral genomes is significantly enhanced by the addition of LLnL/Dox at the time of vector administration. To correlate the level of functional correction with the ability of rAAV vector genomes to express CFTR mRNA, epithelia were harvested following functional analysis and vector-derived mRNA and DNA was quantified for each sample. Quantification of vector derived mRNA was performed as described in Gerard et al. (2003) an RS-PCR method that normalizes the copies of vector-derived and endogenous CFTR mRNAs to the level of β-glucuronidase (GUS) expression (the same method used for analysis of rAAV CF clinical trial sample (see Flotte et al., 2003). Results from this analysis (Figure 8A) demonstrated near undetectable vector-derived CFTR mRNA transcripts in all vector groups that did not receive LLnL/Dox at the time of vector administration. These results support the lack of CFTR functional activity seen in these experimental groups. In contrast, LLnL/Dox treatment significantly enhanced the level of vector-derived CFTR mRNA transcripts in both AV2tgCF and

AV2CF83 vector groups by greater than 150-fold. Although similar levels of induction were seen in AV2/5 vector groups, the total level of vector derived CFTR mRNA remained 10 to 40-fold lower than that seen in the AV2 vector groups. Comparison of transgene derived CFTR mRNA to endogenous CFTR mRNA levels in the various vector groups also reflected greater relative expression in AV2tgCF and AV2CF83 groups (Figure 8B) that was equivalent or slightly greater to that of endogenous levels. Although the fold differences in vector derived CFTR mRNA between the various vector groups did not quantitatively mirror the fold differences in CFTR correction, the overall trends were similar. All rAAV vectors gave much higher levels of vector-derived CFTR mRNA when proteasome modulating agents were applied at the time of infection, and consistent with CFTR functional correction, AAV2 vectors expressed CFTR mRNA at much higher than AAV2/5 vectors.

Current knowledge of the mechanism of proteasome inhibitors to enhance rAAV transduction from the apical membrane suggests that these agents act on intracellular processes that enhance rAAV movement to the nucleus (Duan et al., 2000a; Ding et al., 2003). Based on this underlying hypothesis, the level of viral DNA that remained in cells at 15 days post-transduction for each of the various serotypes and treatment conditions, was assessed. In light of previous studies (Duan et al., 2000a; Ding et al., 2003), it was predicted that LLnL/Dox would predominantly increase the transcriptional activity of viral genomes by virtue of greater nuclear accumulation and conversion to double stranded intermediates. Furthermore, it was predicted that substantial viral DNA would remain in epithelia infected in the absence of LLnL/Dox and that these forms would predominantly be transcriptionally inactive as single stranded genomes. Results from DNA analysis indeed supported these hypotheses. LLnL/Dox treatment only marginally increased DNA persistence by 2-3 fold (Figure 9A) regardless of the vector type. However, when transcriptional activity of viral genomes was assessed by calculating the vector derived mRNA/DNA ratios, LLnL/Dox treatment significantly enhanced the transcriptional activity of vector genomes by 40-50 fold for the AV2 vectors groups (Figure 9B). Enhancement of vector derived mRNA/DNA ratios for the AV5 vector groups were also very large but could not be accurately calculated since mRNA

levels in the absence of LLnL/Dox were at background levels. These findings support the notion that modulation of the proteasome allows for more efficient intracellular processing of rAAV genomes to transcriptionally active intermediates without significantly affecting their overall abundance within cells.

Evaluation of vector derived mRNA/DNA ratios was also useful in assessing increased efficacy of vectors harboring a short 83 bp minimal promoter. Although the CF83 promoter improved gene reporter gene expression in IB3 cells 30-fold above that seen with the ITR promoter (Lynch et al., 1999), the effect on gene expression was minimal in differentiated airway epithelia. RNA/DNA ratios were not significantly different for AV2CF83 as compared to AV2tgCF in the presence of LLnL and Dox, implying no significant augmentation in transcriptional activity of genomes containing the synthetic promoter. However, the RNA/DNA ratios for AV2/5CF83 were approximately 3-fold higher than that for AV2/5tgCF, suggesting that the synthetic promoter may have some beneficial effect on transcription, although not as great as in IB3 cells.

Proteasome modulating agents reduce the amiloride-sensitive sodium currents in CF airway epithelia by decreasing ENaC subunit mRNA levels. ENaC is the major component of baseline short circuit current in CF airway epithelia and is greatly elevated due to a lack of functional CFTR. It has been previously suggested that as little as 6-10% transduction with a CFTR expressing vector can fully correct CFTR-mediated chloride currents in a polarized airway epithelia due to gap-junctional cell-cell coupling of Cl^- ions in the epithelium (Johnson et al., 1992). In contrast, normalization of elevated Na^+ current caused by dysregulated ENaC activity requires 100% transduction of a CF epithelia since CFTR must physically interact with ENaC to properly regulate Na^+ conductance (Johnson et al., 1995). Consequently, the extent of normalization of elevated amiloride-sensitive sodium currents in each of the CFTR vector treated groups could be used to indirectly infer the percentage of cell expressing vector-derived CFTR in the epithelia. Assessment of amiloride-sensitive ENaC short circuit current revealed the surprising finding that all vector groups administered with LLnL/Dox demonstrated complete normalization of elevated Na^+ currents at 15 days post-infection (Figures 10A and 12). Further analysis of this finding demonstrated that this effect on ENaC activity

was independent of vector administration and was also seen in mock-infected controls (Figure 10A). Despite these changes in LLnL/Dox induced ENaC activity, no difference in transmembrane resistance among all groups was seen, suggesting the epithelium had remained intact throughout the experiment. Furthermore, morphologic analysis of 15 days LLnL/Dox treated cultures reveled no obvious morphologic changes in epithelial integrity as compared to control untreated cultures. This findings supports the notion that down-regulation of ENaC in these studies was independent of CFTR correction and predominantly mediated by the proteasome modulating agents.

Intrigued by the finding that a single treatment with LLnL/Dox could normalize ENaC currents in CF airway epithelia for 15 days, it was hypothesized that the mechanism may involve down regulation of certain ENaC subunits. To this end, quantitative TaqMan RT-PCR was used to determine the mRNA levels of α, β, and γ ENaC subunits in polarized CF airway epithelia following LLnL/Dox treatment (Figure 10B). Results from this analysis demonstrated that the ratio of γ-ENaC subunit to β-actin mRNA was most significantly decreased (16-fold) in the LLnL/Dox treatment group (0.014+/-0.0046, n=9) as compared to the non-treated control (0.222+/-0.096, n = 12). Similarly, the mRNA level of α-ENaC and β-ENaC were also reduced by 2 and 3-fold, respectively, following treatment of proteasome modulators. These findings suggested that decreased transcription of predominantly the γ-ENaC subunits might be responsible for the observed inhibition of ENaC currents by LLnL and/or Dox.

To distinguish whether LLnL and/or Dox were acting to inhibit ENaC function in CF airway epithelium, the effect of each compound individually was analyzed. Results from this analysis demonstrated that Dox alone provided equivalent inhibition of ENaC Isc as seen with combined Dox/LLnL and was greater than LLnL alone (data not shown). With the hypothesis that transcriptional inhibition of ENaC subunit genes was accounting for the Dox-dependent decline in ENaC function, the time course of changes in amiloride-sensitive Isc seen in the presence of Dox was evaluated. Because this study required a relatively large number of CF samples, a recently reported transformed CuFi cell model was used to

generate polarized air-liquid interphase CF epithelia (Zabner et al., 2003). This model has previously demonstrated elevated baseline amiloride-sensitive Isc indicative of CF-associated elevated ENaC activity. Results from this analysis demonstrated a gradual decline in ENaC Isc of Dox-treated CuFi epithelia over the course of 1 to 14 days that was not observed in control untreated samples (Figure 10C).

Doxorubicin treatment increases γ-ENaC promoter methylation. Since it has been reported that doxorubicin treatment leads to CpG demethylation of MDR-1 gene promoter and a consequent increase in MDR expression (Kusaba et al., 1999), it was hypothesized that increased CpG methylation of the γ-ENaC gene promoter might produce the opposite Dox-dependent effect. To test this hypothesis, an airway cell model system for which genomic DNA could be easily generated was developed. First, it was determined whether a non-CF airway cell line (A549) produced similar regulatory changes in γ-ENaC mRNA expression following Dox treatment. Indeed, Dox treatment of A549 cells led to a dramatic decrease in γ-ENaC/β-actin mRNA ratios in cultures grown to confluence (Figure 11A). Whereas untreated A549 cells progressively increased γ-ENaC mRNA levels at post-confluency, γ-ENaC mRNA levels remained consistently low in Dox treated cultures before and after confluence.

Previous reports analyzing the γ-ENaC gene promoter have demonstrated the existence of a fairly extensive CpG island at approximately –200 to –340 bp (Auerbach et al., 2000). Upon further analysis, a second large CpG island, located approximately 1.8 kb upstream of the transcriptional starting site of the γ-ENaC gene, was identified using a online tool CpG Island Searcher, http://www.methdb.de/ (Figure 11B). Using a methylation-sensitive endonuclease digestion PCR assay (Nakayama et al., 1998), genomic DNA from A549 cells treated with or without Dox, was assessed for CpG methylation. This assay utilized primers flanking a 310 bp region in this CpG island that contained multiple MspI/HpaII sites. MspI and HpaII digest the same sequence in DNA, however, HpaII will not digest if CpG methylation is present. Results from this analysis demonstrated a significant Dox-dependent protection from HpaII digestion at this

region of the CpG island (Figure 11C). These findings suggested that CpG methylation of the γ-ENaC promoter indeed occurs in response to Dox treatment.

Discussion

Current knowledge rAAV transduction biology suggests that both receptor abundance and/or intracellular barriers that affect the movement of virus to the nucleus are significant determinants that influence efficiency of this vector for gene therapy in a given tissue target. Currently gene therapy trials for CF lung disease have demonstrated an impressive safety profile for rAAV-2, but failed to demonstrate CFTR transgene expression (Flotte et al., 2003). The source of this shortcoming can be due to several aspects of rAAV biology and/or vector design. First, since the CFTR cDNA is very large, low-level expression in current trials may be due to low promoter activity of the ITR used to drive expression of the CFTR gene. Second, studies in mice have demonstrated that rAAV-5 much more efficiently infects airway epithelia (Zabner et al., Auricchio et al., 2002). Hence, the choice of rAAV-2 as a therapeutic platform for lung gene delivery may be suboptimal. Third, studies have also demonstrated that intracellular processes controlled by the ubiquitin/proteasome system significantly influence rAAV transduction with both type 2 and 5 serotypes (Duan et al., 2000; Ding et al., 2003). The present study was designed to directly evaluate which of these three parameters has the greatest influence on the efficacy of current CF lung gene therapy efforts. As such, the current clinically used CFTR AAV-2 vector was compared to both AAV-2/5 pseudotyped virus and a new vector design which incorporates a minimal 83 bp promoter upstream of CFTR.

An important initial reference to current clinical trails was the assessment of CFTR delivery and expression in the absence of applied proteasome inhibitors. In this context, ITR promoter rAAV-2 and rAAV-2/5 based vectors gave no significant CFTR functional correction or mRNA expression, but were capable of delivering a significant number of viral genomes into airway epithelia that persisted for 15 days. For AV2tgCF vectors, this finding is consistent with current clinical findings (Flotte et al., 2003). Co-administration of proteasome modulating agents significantly improved the ability of both rAAV-2 and rAAV-2/5 vector to correct CFTR chloride transport abnormalities. Importantly, this is the first demonstration of

CFTR functional correction in polarized human CF airway epithelia. Surprising, based on mouse studies, rAAV-2 vectors gave significantly higher levels of CFTR functional correction and mRNA expression than compared to rAAV-2/5. Additionally, the addition of the CF83 minimal promoter demonstrated only marginal improvement in functional correction and/or CFTR mRNA expression.

Several important findings from these comparisons have important implications on our understanding of rAAV transduction biology in the human airway. First, although proteasome modulators dramatically increased CFTR mRNA expression and functional correction, they only marginally increased vector genome persistence in cells. Such findings are consistent with previous work demonstrating that intracellular processing of virus genomes is the major rate-limiting step in airway transduction. Based on previous reports, this rate-limiting step appears to involve trafficking of virus to the nucleus in a ubiquitin/proteasome dependent manner and not second strand synthesis of viral genomes (Duan et al., 2000a; Ding et al., 2003). In support of this mechanism, the functional activity of viral genomes (mRNA/vector DNA ratios) increased 40-50 fold following LLnL/Dox treatment. Although rAAV-2 vectors performed better than rAAV-5 vectors, the effect of proteasome modulating agents on increasing the functional activity of viral genomes was universal to both type 2 and type 5 serotypes.

One of the most surprising findings from the current analysis of proteasome modulating agents to enhance rAAV-mediated CFTR delivery to the CF airway, was the observation that these same agents simultaneously normalized CF-associated ENaC hyperactivity through an independent mechanism. Such a finding suggests that proteasome modulating agents may have dual therapeutic utility as pharmacologic agents to treat primary pathology and enhance gene therapy for CF lung disease. The amiloride-sensitive epithelial sodium channel (ENaC) controls sodium transport across many types of epithelia, including airway, kidney and colon. In the CF airway, the loss of CFTR function results in uninhibited excessive ENaC activity, which in turn has been hypothesized to dehydrate airway surface and diminish the cell surface clearance ability of airway (Johnson et al., 1995), leading to severe recurrent airway infections in CF patients (Jiang et al., 1998; Guggino et

al., 1999). In the current study, the combined administration of LLnL and doxorubicin (Dox) inhibited the enhanced ENaC activity seen in CF epithelial in a manner that was independent of CFTR complementation. This affect on ENaC activity appears to be predominantly due to altered gene transcription of the γ-ENaC promoter leading to reduced levels of γ-ENaC mRNA.

ENaC activity can be regulated either by altering the channel open probability or the number of functional ENaC molecules on cell surface. Previous studies have demonstrated a link between the ubiquitin-proteasome proteolytic system and regulation of ENaC turnover at the cell surface. ENaC consists of three subunits (α, β, and γ) each of which has a proline rich region (PPXY) at the C-terminal end. The ubiquitin ligase Nedd4 interact with ENaC through this PPXY region and mutation of a group of lysine residues at the N-terminus of the α and γ subunits leading to inhibition of ubiquitination and increased ENaC activity (Staub et al., 1997). Additionally, inhibition of proteasome activity by carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal (MG132) has been shown to increase the level of ENaC subunits at the membrane and ENaC activity (Malik et al., 2001). These findings are in stark contrast to the present observations that doxorubicin inhibits long-term ENaC activity through increases in γ-ENaC promoter CpG methylation and potentially also other ENaC subunit promoters. Furthermore, the prolonged effects of a single administration of LLnL/Dox that last for 15 days supports an inhibitory mechanism involving transcriptional regulation that is likely different than the short-term regulation of ENaC by Nedd4 ubiquitin ligase. Interestingly, doxorubicin has been shown to alter methylation of the MDR-1 promoter and increase transcriptional activity of the multi-drug resistance gene (MDR-1) (Kusaba et al., 1999; Nakayama et al., 1998; Ando et al., 2000). Although the net effect of doxorubicin on MDR-1 and γ-ENaC promoter methylation are opposite, the processing controlling changes in CpG methylation may be similarly regulated.

The finding that proteasome modulating agents alter baseline ENaC activity in CF airway epithelia may have practical therapeutic applications outside their combined ability to also enhance rAAV transduction. Given the fact that ENaC hyperactivity is thought to dehydrate the surface airway fluid layer in the CF and

decrease airway clearance of bacteria, inhaled proteasome modulating agents that inhibit ENaC activity could be applied as an aerosolized compound(s) to the lungs of CF patients to enhance airway clearance. Although previous attempts to inhibit ENaC using aerosolized amiloride (an agent that binds directly to cell bound ENaC), have shown little functional benefit in CF clinical trials (Knowles et al., 1999; Graham et al., 1993), it is possible that such earlier approaches have failed due to the short half-life of the inhibitory compound. Since doxorubicin demonstrates a much longer inhibitory effect on ENaC activity, its efficacy may be significantly improved. Thus, the present findings suggest that the current clinically used rAAV-2 CFTR vector may possess substantial therapeutic utility for gene therapy of CF lung disease if proteasome modulating agents are simultaneously administered at the time of infection. The dual therapeutic utility of pharmico-gene therapy agents to both treat primary pathophysiologic defects of a disease while simultaneously enhancing the efficacy of gene therapy represents a new area for drug development.

Example 9

Assays for Agents that Alter ENaC Transcription

Agents that affect ENaC transcription can be evaluated using a variety of assays, including *in vitro* assays such as those which measure transcription or RNA stability, e.g., RT PCR for the various subunits, of ENaC following treatment of A549 cells or other suitable cell types with one or more agents. For instance, cells, lysates thereof or *in vitro* transcription/translation mixtures, are placed in a 96 well plate format followed by high throughput Real Time PCR for one or more ENaC subunits in the 96 well plate. In one embodiment, the cells may be infected with a viral vector prior to, during or after one or more of the agents are added to cells, lysates thereof or *in vitro* transcription/translation mixtures.

In vivo based screening for function inhibition of ENaC using CF mice may also be employed. Electrophysiologic properties of CF mouse nasal epithelium are very similar to those found in the human nasal and lung airways (Grubb et al., 1994), and defective CFTR leads to hyperactivity of ENaC. To confirm the ability of agents identified in cell based screens to inhibit ENaC transcription and hence

ENaC function, CFTR knockout mice may be employed. Agents to be tested are delivered to mice by the appropriate route including but not limited to i.v., i.p., endotracheal or nasal application at an interval (days to weeks) prior to functional analysis of nasal potential difference measurements. Briefly, a 200 μm internal diameter catheter was linked directly to a perfusion syringe pump. The syringe was linked via a 1 M KCL agar salt bridge to a calomel electrode and a voltmeter. The second calomel electrode was linked to a 1 M KCL agar-filled 21 gauge needle implanted subcutaneously in the mouse. Once the catheter was placed in the nasal cavity, perfusion was initiated at a flow rate of 2 μl/min. The position of the catheter was adjusted to obtain the most negative transmembrane potential in the initial starting buffer. Potential difference measurements were made with a sequence of perfused buffers as follows: i) Hepes phosphate buffered Ringer's solution (HPBR) containing 10 mM Hepes pH 7.4, 145 mM NaCl, 5 mM KCL, 1.2 mM MgSO4, 1.2 mM Ca-gluconate, 2.4 mM K2HPO4, 0.4 mM KH2PO4, ii) HPBR with 100 μM amiloride, iii) HPBR chloride free (using gluconate in place of chloride), 100 μM amiloride, iv) HPBR chloride free, 100 μM amiloride, 10 μM forskolin, 100 μM UTP. Computer-assisted recording of mVs was taken every five seconds for a total of 3 minutes for each buffer.

The affect of drug pretreatments on the amiloride sensitive response is evaluated. In the case of doxorubicin, it is expected that the amiloride-sensitive voltage change would be reduced in CF mice as compared to vehicle treated controls. Assessment of low chloride, forskolin, and UTP responses serve as controls for the integrity of the epithelium. To confirm changes in ENaC transcription, DNA is isolated from nasal cells and methylation sensitive PCR analysis performed for the various ENaC subunit promoters. An example of such nasal potential difference (PD) measurements is given in Figure 21.

Example 10

In vitro and *In vivo* Activities of Additional Proteasome Modulators

Based on results with doxorubicin, a small number of FDA approved anthracyclines were tested for their relative *in vitro* and *in vivo* activities on AAV transduction. HeLa cells were infected with 100 ppc AAV2FLAG-Luc for 2 hours

in the presence of different anthracyclines, e.g., doxorubicin, daunarubicin (Cerubidine), epirubicin (Ellence™), and idarubicin (Idamycin®), and cells harvested 48 hours later. The anthracyclines were pharmaceutical grade, and prepared according to the manufacturer's instructions. Prior to use, the agents were diluted in sterile water to an equal mass, e.g., 0.6 μg/mL, 3 μg/mL and 6 μg/mL. The results are shown in Figure 14. For example, 3 μg/mL idamycin increased luciferase expression by over 5000-fold while doxorubin increased luciferase expression by 58-fold. Generally, the potency was as follows: idarubicin > daunarubicin > epirubicin > doxorubicin.

Six groups of ten, five-to-seven week-old, Balb/c mice (5 male and 5 female per group) were employed in a comparison of the relative *in vivo* potency and safety of different anthracycline derivatives at a single dose after intranasal delivery. Treatment was administered as shown in Table 3. Animals were followed for seven days post dose.

Table 3

Group	R_x	rAAV Treatment (Dose in DRP)	Proteasome Modulator	Proteasome Modulator Dose (% of HDE)	Route of Administration (rAAV/Inhibitor)	Day of Sacrifice
1	No R_x control	Vehicle	Vehicle	0	Intranasal/ Intranasal	7
2	Vector control	1×10^{12} AAV2-GFP + 1×10^{11} AAV2-Luc	Vehicle	0	Intranasal/ Intranasal	7
3	Test 1	1×10^{12} AAV2-GFP + 1×10^{11} AAV2-Luc	Doxorubicin	10	Intranasal/ Intranasal	7
4	Test 2	1×10^{12} AAV2-GFP + 1×10^{11} AAV2-Luc	Idamycin	10	Intranasal/ Intranasal	7

Group	R_x	rAAV Treatment (Dose in DRP)	Proteasome Modulator	Proteasome Modulator Dose (% of HDE)	Route of Administration (rAAV/Inhibitor)	Day of Sacrifice
5	Test 3	1×10^{12} AAV2-GFP + 1×10^{11} AAV2-Luc	Doxil	10	Intranasal/ Intranasal	7
6	Positive control	1×10^{12} AAV2-GFP + 1×10^{11} AAV2-Luc	Doxil	75	Intranasal/ Intravenous	7

The dose of modulator was based on the Human Dose Equivalent (HDE) and is summarized below in Table 4. For intranasal dose administration, the dose was held constant at 10% of the HDE. For the intravenous positive control (Doxil), a dose of 10 mg/kg (75%) of the HDE was used. This represented the lowest dose that gave a 10% increase in mean and median luciferase expression in earlier studies.

Table 4. Human dose equivalent calculations

Drug	Drug Concentration (mg/mL)	Human dose (mg/m^2)	10% Human Dose (mg/m^2)	10% of Human dose in mg/kg for a mouse (dose $mg/m^2/3$)	10% of human dose per 20 gram mouse (mg)	Volume of stock drug (mL) per mouse
Adriamycin	2	40-75	7.5	2.5 mg/kg	0.05 mg	0.025 mL
Idamycin	1	10-12	1.2	0.4 mg/kg	0.008 mg	0.008 mL
Doxil	2	10-40	4.0	1.3 mg/kg	0.026 mg	0.013 mL

Dose calculation: Animal (mouse) dose in mg/kg x 3 (mouse km) = dose in mg/m^2. mg per mouse = Dose in mg/kg x 0.02 kg mouse

Safety endpoints included morbidity and mortality, clinical observations, body weights, gross necropsy observations and histopathology. Transduction endpoints included luciferase and GFP analysis.

On the day of sacrifice, the left lung was clamped off at the level of the extrapulmonary bronchi, removed and frozen on dry ice. The left lung was homogenized and processed for luciferase expression using Promega's luciferase assay

system (Madison, WI). Luminescence was measured using the Berthold AutoLumat LB953 instrument. Samples were normalized for total protein using Pierce's Coomassie Plus Protein Assay Reagent (Rockford, IL).

Intranasal administration of doxorubicin and idamycin at 10% HDE were both associated with early mortality of some animals, ruffled hair coats and sick mice. In addition, those animals that survived also lost considerable body weight over the week. The intranasally doxil treated mice did better than the doxorubicin- or idamycin-treated animals in that there was no early mortality and they appeared clinically normal. However, they also lost weight. The intravenously doxil treated mice fared the best.

Intranasal treatment of doxorubicin and idamycin resulted in increased luciferase expression (Figure 15 and Table 5). Treatment with doxil at a 10% HDE (both intravenously and intranasally) resulted in an average increase in luciferase expression by 49- and 74-fold, respectively, 7 days post-dose.

Table 5. Fold increase in luciferase expression

Rx	Average	Standard Deviation	Fold Increase
Vehicle – M	1.28E+03	4.05E+02	
Vehicle – F	1.32E+03	6.64E+02	
Vehicle	1.30E+03	5.19E+02	
No Rx – M	7.28E+03	5.01E+03	1
No Rx – F	3.56E+03	1.27E+03	1
No Rx	5.63E+03	4.12E+03	1
Doxorubicin (10% HDE) – M	*4.21E+06	*2.06E+06	578
Doxorubicin (10% HDE) – F	5.44E+05	4.00E+05	153
Doxorubicin (10% HDE)	1.77E+06	2.13E+06	314
Idamycin (10% HDE) - M	8.11E+05	2.81E+05	111
Idamycin (10% HDE) - F	2.02E+05	1.05E+05	57
Idamycin (10% HDE)	5.06E+05	3.80E+05	90
Doxil (10% HDE) - M	6.68E+05	2.57E+05	92
Doxil (10% HDE) - F	1.65E+05	7.15E+04	46
Doxil (10% HDE)	4.16E+05	3.19E+05	74

Doxil (75% HDE iv) - M	3.16E+05	2.69E+05	43
Doxil (75% HDE iv) - F	2.31E+05	1.21E+05	65
Doxil (75% HDE iv)	2.73E+05	2.02E+05	49

*Average and standard deviation were calculated from two numbers

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification, this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details herein may be varied considerably without departing from the basic principles of the invention.